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Effect of Delvinal Sodium on Respiratory Metabolism of the Rhesus Monkey (*Macaca mulatta*).*

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Cope and Hancock[1] synthesized 5-ethyl-5-(1-methyl-1-butenyl) barbituric acid and reported it to be an hypnotic. The development of tolerance to the sodium salt of this drug (delvinal sodium) has been noted in experimental animals by Carmichael and Thompson[2] and clinically by Davidoff.[3] Hendrix,[4] who has also used the delvinal sodium clinically, reports that the drug reduced the basal metabolic rate of white rats 15.7% as determined by the method of Richards and Collison.[5]

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* Aided by a research grant from the University of Alabama.

[1] Cope, A. C., and Hancock, E. M., *J. Am. Chem. Soc.*, 1939, **61**, 776.

[2] Carmichael, E. B., and Thompson, W. D., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 233.

[3] Davidoff, E., *Psychiatric Quarterly*, 1941, **15**, 370.

[4] Hendrix, J. P., *J. Pharm. and Exp. Therap.*, 1940, **68**, 22.

In continuation of our experiments with delvinal sodium, we thought that it would be of interest to determine its effect on the basal metabolic rate of rhesus monkeys *(Macaca mulatta)*, since this drug is being used clinically. A new method, which was devised by one of us (S.A.P.),[6] was used. The apparatus provides a continuous photographic record of the rate of O_2 consumption from which the metabolic rate at any instant can be calculated.

Ten normal monkeys were used in this series of experiments. The diet for the last meal, previous to performing an experiment, consisted of fruits and vegetables (apples, bananas, oranges, cabbage, lettuce, and tomatoes). The feeding took place between 4:00 p.m. and 5:00 p.m.; then the monkeys were allowed to fast until the end of the experiment the next afternoon. All tests were begun between 9:40 a.m. and 10:40 a.m. and due to the character of the last meal, the animals were very probably postabsorptive about 12 to 15 hours before the basal heat production was determined.

The monkeys were weighed and placed in the chamber to determine the O_2 consumed. This preliminary control period ranged from 1.5 hours to 4 hours, in order to obtain a record free from activity. Then the animal was removed from the chamber and weighed again before it was injected intraperitoneally with a freshly prepared 2% solution of delvinal sodium. When anesthesia set in, the animal was placed back in the chamber and a record was again made of his O_2 consumption. This experimental period varied from 2.5 hours to 5.5 hours. The depth of narcosis was determined on removal from the chamber and at intervals thereafter. The rectal temperature was taken before and after the control period and after final removal from the chamber. The bath which surrounded the chamber varied from 25.4° to 28°C during the control period and from 26.3° to 29.2°C when the anesthetized animal was in the chamber.

The surface area was calculated from the Meeh[7] formula $S = KW^{2/3}$. The value for K was determined by Lee and Fox[8] to be 11.7 for rhesus monkeys. A respiratory quotient of 0.82 was assumed arbitrarily and the caloric value for one liter of oxygen was taken as 4.825 calories. The average value is only about 11% higher

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[5] Richards, A. N., and Collison, L. W., *J. Physiol.*, 1928, **66**, 299.

[6] Peoples, S. A., *Science*, 1941, **94**, 373.

[7] Meeh, K., *Z. f. Biol.*, 1879, **15**, 425.

[8] Lee, M. O., and Fox, E. L., *Am. J. Physiol.*, 1933, **106**, 91.

TABLE I.

Respiratory Metabolism of Normal Rhesus Monkeys Before and After Administration of Delvinal Sodium.

	Control period				Experimental period						On removal from chamber		
								% change in cals./sq.m./hr at the following intervals after injection with delvinal sodium					
Animal No.	Wt in kg	Rectal temperature	Cals./sq.m./hr	Rectal temperature after	Wt in kg	Dose in mg/kg	Degree of narcosis when placed in chamber	120 min	150 min	180 min	Rectal temperature	Degree of narcosis	Wt in kg
27	3.759	103.6	31.1	102.8	3.645	35	DSA	—18	—19	—	101.2	LSA	3.607
28	4.716	102.5	29.9	101.9	4.665	30	LSA	—18	—16	—16	101.0	VLA	4.642
30	4.335	103.8	32.5	102.9	4.250	35	LSA	—12	— 9	—10	102.4	LSA	4.217
33	3.161	103.6	28.7	102.1	3.117	40	DSA	—11	—13	—17	99.6	DSA	3.102
35	2.795	103.7	29.9	102.3	2.757	40	DSA	—34	—35	—36	97.8	DSA	2.743
36	3.077	102.4	26.4	102.0	3.003	40	LSA	—25	—25	—26	98.4	DSA	2.993
38	2.649	102.7	30.9	102.2	2.615	40	DSA	—31	—32	—31	100.2	DSA	2.603
43	3.907	103.1	33.2	103.0	3.819	40	LSA	—15	—16	—11	102.9	LSA	3.794
44	3.036	103.1	32.6	102.7	2.998	45	DSA	— 8	— 7	— 7	101.2	DSA	2.986
48	2.830	103.0	29.5	101.9	2.788	45	DSA	—21	—22	—23	97.8	DSA	2.768
Average		103.1	30.46	102.3				—20.3	—19.4	—20	100.2		

than those reported by Bruhn[9] and about 20% higher than those reported by Rakieten.[10] The monkeys were not trained in the apparatus and none of the experiments were performed at night as suggested by Bruhn[9] so that the control values may be slightly higher than the true basal rate.

The data that were obtained during the control period and during the period following the administration of the delvinal sodium, are shown in Table I. The metabolic rate changed so slowly during the narcosis resulting from the delvinal sodium that the instantaneous values at the arbitrary times selected give a graphic picture of the smooth curve obtained. The degree of anesthesia was difficult to judge but the following criteria were adopted: (1) deep surgical anesthesia when there was no response to any stimuli, (2) light surgical anesthesia when there was reflex response to a severe pinch in the flank, and (3) very light anesthesia when there were no voluntary movements but when a painful stimulus temporarily caused coordinated movements.

It is interesting to note that there was a significant decrease in the oxygen consumption in every animal that received delvinal sodium. However, this decrease in oxygen consumption does not seem to be related to either the dose of the drug or the drop in rectal temperature during the experimental period.

Summary. The average basal heat production of ten normal rhesus monkeys was 30.46 cal/square meter/hour. The O_2 consumption was decreased by doses of 30 mg to 45 mg of delvinal sodium/kilo of monkey. The 40 mg and 45 mg doses/kilo produced surgical anesthesia that lasted for about 3 hours to 5 hours.

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Blood Enzymes after Ligation of all Pancreatic Ducts.*

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Ligation of the pancreatic ducts of dogs is soon followed by a rise in the concentration of blood amylase which has been assumed by most workers in this field to return to the previous level within 10 days to a few weeks, with occasional transient rises up to the sixth week.[1] Golden, Sieracki, Handelsman and Pratt[2] reported that following ligation and section of all pancreatic ducts, a permanent increase of blood amylase resulted in 3 out of 4 dogs. Autopsy showed the pancreas to be transformed into a small thin, cordlike mass in all 4 dogs. None of their animals had been suffering from nephritis.[2, 3] Popper and Plotke[4] in recently published animal experiments, demonstrated that an artificially raised blood enzyme level falls to normal within a few hours, if no further inflow of enzymes into the circulation takes place. Since the pancreatic enzymes are produced only in the acinar tissues of the pancreas, it is difficult to understand how blood amylase can remain at an increased level in case of a complete atrophy of the acinar tissues. It is known, of course, that the pancreas is not the sole source of blood amylase, and following total pancreatectomy blood amylase soon returns to its control level. In case of increased blood amylase, however, no other source is known than the pancreas, or, rarely, the salivary glands.[5]

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[9] Bruhn, J. M., *Am. J. Physiol.*, 1934, **110**, 477.

[10] Rakieten, N., *J. Nutrition*, 1935, **10**, 357.

* Aided by the John D. and Fannie K. Herz Fund.

In order to explain the above, the pancreas of 4 dogs was isolated from the duodenum in exactly the same way as Golden and collaborators have reported. All connecting strands were ligated and divided and only the necessary blood vessels remained intact. Omentum was interposed between duodenum and pancreas to avoid the possibility of recanalization. Two of the 4 dogs died from pancreatitis. The remaining 2 were kept alive for 12 months. One of them lost weight slowly but continually. The other dog retained its original weight until 9 months after operation, when it began to drop suddenly. Both dogs died 12 months after operation from an intercurrent disease, and a thorough necropsy was performed. In both animals a small, thin, cordlike mass was found, which did not resemble the pancreas at all. Microscopic examination showed an abundance of connective tissue, containing many nerve fibers, blood vessels and islets of Langerhans. In a few places small nests of cells were found which seemed to be acinar tissue.

Table I shows the enzyme concentration in the blood of the 2 dogs before and after operation. Blood amylase was determined by the

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[1] Gould, L. K., and Carlson, A. J., *Am. J. Physiol.*, 1911-12, **29**, 165.

[2] Golden, L. A., Sieracki, L. A., Handelsman, M. B., and Pratt, J. H., *Am. J. Dig. Dis.*, 1939, **6**, 327.

[3] Gray, S. H., and Somogyi, M., PROC. SOC. EXP. BIOL. AND MED., 1932, **29**, 1126.

[4] Popper, H. L., and Plotke, F., *Surgery*, 1941, **9**, 706.

[5] Popper, H. L., Hirschhorn, S., and Selinger, A., *Klin. Wochen.*, 1931, **10**, 493.

Wohlgemuth method; normal values for the dog range between 32 and 128 Wohlgemuth units. Blood lipase was determined, using Crandall and Cherry's method[6] and the values are expressed in cc of N/20 NaOH, 1cc being equal to one unit. Lipase values up to 1.0 cc are normal in dogs, according to our experience.

Dog 1 showed a marked increase of blood amylase 24 hours after ligation of the pancreatic ducts. The next enzyme determination, performed 11 days after the operation, revealed normal blood amylase which did not change during the following 12 months of observation. Lipase was not found changed, but it is possible that a rise of lipase had taken place between the 1st and 11th postoperative day, since no lipase determination was performed in this period.

Dog 2 showed a marked increase of blood amylase 1 day after the operation, and 6 days after operation still a slight increase. The next

TABLE I.
Blood Enzymes Following Ligature of All Pancreatic Ducts.

Time	Wohlgemuth units Amylase	cc N/20 NaOH Lipase	Weight, lbs
Dog 1			
Before operation	64	.2	41
After operation			
1 day	1024	.2	
11 ”	32	.4	
3 wk	64	.7	28
6 ”	128		26½
9 ”	128	.3	25½
11 ”	64	.7	
14 ”	128	.2	27
20 ”	128	.8	25
24 ”	128	.4	22½
7 mo	128		
8 ”	64	.8	
10 ”	64	.4	20
11 ”	64	.6	17
Dog 2			
Before operation	128	.6	35
After operation			
1 day	1024	.4	
6 ”	256	.8	
23 ”	128	.3	32
6 wk	1024	3.1	32
8 ”	128	.8	
11 ”	512	.9	
13 ”	512	.4	36½
17 ”	128	.8	36
21 ”	128		34
6 mo	64		
7½ ”	64	.8	33
9 ”	64	.6	33
10½ ”	64	.6	28
12 ”	64	.2	22

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[6] Crandall, L. A., Jr., and Cherry, I. S., *Am. J. Physiol.*, 1932, **100**, 266.

determination, performed on the 23rd postoperative day, revealed a normal concentration of blood amylase. In the 6th postoperative week a marked rise of amylase was again found, but after 8 weeks a normal level was present. From the 11th to the 13th week, a third rise was observed. From then on its concentration remained permanently below the preoperative level. Blood lipase showed no increase following the operation, but a marked rise accompanied the secondary rise of amylase in the 6th postoperative week. Further determinations revealed values within normal limits. Our observations on blood lipase confirm similar ones by Chiray, Berdet and Taschner.[7]

Antopol, Schifrin and Tuchman[8] and Friedman and Thompson[9] have shown that injection of Mecholyl[†]-eserine is followed by an increase of blood amylase provided that functioning pancreatic tissue is present, and we[4] have reported similar results on blood lipase. We have tried, therefore, to stimulate the pancreas of our experimental animals with Mecholyl-eserine, 24 and 21 weeks respectively after ligation of the pancreatic ducts. Neither an increase of amylase nor of lipase occurred, suggesting the absence of functioning pancreatic tissue.

Our findings are in line with our present knowledge about blood amylase. An increase occurs following retention of the external pancreatic secretion and Hess[10] has shown that the congested pancreas ceases secreting very soon. Following this, no further absorption of enzymes into the blood circulation takes place and the increased blood enzyme level will drop within a very short time as we have demonstrated recently.[4]

The process of secretion by the pancreas and subsequent absorption of enzymes may repeat itself and may last a certain time. It is certainly to be thought of not only as a simple mechanical process, but also due to inflammatory reactions of the pancreas. However, after a few months the atrophy of the pancreas is completed, the acinar tissue has disappeared, and no further production and consequent absorption of enzymes can take place. Yet, blood amylase does not disappear entirely but remains at a low level which, according to observations on pancreatectomized dogs, must be derived from other sources.

It is obvious that drugs, increasing the concentration of blood enzymes by stimulation of the pancreas cannot cause any change at this stage.

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[7] Chiray, M., Berdet, H., and Taschner, E., *Arch. d. mal. de l'app. digestif*, 1931, **21**, 1137.

[8] Antopol, W., Schifrin, A., and Tuchman, L., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 383.

[9] Friedman, I., and Thompson, W. R., *Ann. Surg.*, 1936, **104**, 388.

[†] Acetyl-beta-methylcholine HCl

[10] Hess, O., *Grenzgebiete der Medizin und Chirurgie*, 1909, **19**, 637.

Summary. In 2 dogs separation of the pancreas from the duodenum with permanent occlusion of all pancreatic ducts was followed by a transient increase of blood amylase. No permanent increase of blood amylase was found as has been reported by others. The dogs showed no increase of blood lipase after the operation. One of these dogs had a secondary and tertiary rise of blood amylase as described by Gould and Carlson, and a transient rise of blood lipase coincident with the secondary rise of amylase.

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Sulfonamide Compounds in Treatment of Experimental *B. typhosus* (*Eberthella typhosus*) Infections of Rabbits.

JOHN A. KOLMER AND ANNA M. RULE.

From the Research Institute of Cutaneous Medicine, Philadelphia, Pa.

Buttle, Parish, McLeod and Stephenson[1] found that p-aminobenzenesulfonamide protects mice against multiple lethal doses of *B. typhosus* and *B. paratyphosus B.* Kolmer and Rule[2] also observed that sulfanilamide administered by subcutaneous and intraperitoneal injection was slightly effective in the treatment of mice inoculated intraperitoneally with *B. typhosus* but noted that sulfapyridine was less effective.

In the present investigation sulfanilamide, sulfapyridine and sulfathiazole were employed by oral administration in the treatment of rabbits inoculated intravenously with *B. typhosus.* The strain employed was of such virulence that the intravenous injection of 0.6 cc of 24-hour broth culture per kilo produced a rapidly fatal infection. Since it was desired to produce a prolonged infection for treatment purposes rabbits were given 0.2 cc of culture per kilo intravenously at daily intervals for 6 days. As a general rule 3 inoculations at daily intervals produced fatal infections with positive heart blood cultures and this amount was employed in order to avoid overwhelmingly severe infections and to permit the administration of multiple doses of the compounds.

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[1] Buttle, G. A. H., Parish, H. J., McLeod, M., and Stephenson, D., *Lancet,* 1937, **1**, 681.

[2] Kolmer, J. A., and Rule, A. M., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 615.

Each compound was given orally in doses of 0.1 and 0.2 g per kilo twice daily for 8 days to surviving animals. The first dose of each was administered 2 hours after the first inoculation of culture and the second 4 hours later, followed thereafter by 2 doses daily (10 a.m. and 3 p.m.). The subsequent daily inoculations with *B. typhosus* were 2 hours before the morning doses of compounds. Heart blood cultures were made daily.

The results are summarized in Table I. Of 16 untreated controls 4, or 25%, survived, indicating that the amount of culture employed was about the minimal lethal dose and therefore favorable for eliciting any therapeutic effects on the part of the compounds employed. It will be observed that of 12 rabbits given sulfanilamide in dose of 0.1 g per kilo 8, or 66.6%, survived while 4 (33.3%) survived with this dose of sulfapyridine and 4 (33.3%) with sulfathiazole. In other words, sulfanilamide was more effective than sulfapyridine as observed in our previous investigation employing mice.[2]

With sulfanilamide in dose of 0.2 g per kilo all of the animals survived; the same was observed with sulfapyridine but only 4 (33.3%) survived in the case of sulfathiazole. It would appear, therefore, that these sulfonamide compounds possess some therapeutic activity in the treatment of rabbits inoculated intravenously with virulent *B. typhosus* but that the effects are slight, as likewise found in the treatment of mice inoculated intraperitoneally. Of the 3 compounds employed sulfanilamide was found most effective, followed in order by sulfapyridine and sulfathiazole.

Apparently recovery of the surviving rabbits was aided by the rapid production of antibodies as the sera of all surviving animals,

TABLE I.
Sulfonamide Compounds in Treatment of Experimental *B. typhosus* Infections of Rabbits.*

Compounds†	Dose per kg (g)	No. animals	Survivals; Days								% survivals
			1	2	3	4	5	7	10	14	
Sulfan.	0.1	12	12	12	12	12	8	8	8	8	66.6
”	0.2	12	12	12	12	12	12	12	12	12	100
Sulfapyr.	0.1	12	12	12	12	8	4	4	4	4	33.3
”	0.2	12	12	12	12	12	12	12	12	12	100
Sulfathiazole	0.1	12	12	8	4	4	4	4	4	4	33.3
”	0.2	12	12	12	6	4	4	4	4	4	33.3
Controls	—	16	16	16	12	8	8	4	4	4	25

*Inoculated intravenously with 0.2 cc of 24-hr broth culture per kg daily for 6 days.

†Twice daily (10 a.m. and 3 p.m.) for 8 days in succession.

including the 2 controls, showed the presence of H agglutinins for the strain employed in final dilution ranging from 1:2560 to 1:5120 when tested about 2 weeks after the termination of the experiment. Agglutination of O antigen ranged from 1:10,240 to 1:40,960 with all of the sera tested.

Of the 16 untreated controls 12 showed positive blood cultures at daily intervals up to the time of death; blood cultures of the 4 surviving animals were negative throughout. Of the 72 treated animals positive blood cultures were observed at daily intervals up to the fourth day of treatment in 50 or 70%; thereafter they were sterile in the case of those that subsequently succumbed, as likewise in those surviving.

Summary. (1) Sulfanilamide, sulfapyridine and sulfathiazole were slightly effective in the treatment of *B. typhosus* septicemia of rabbits. (2) Sulfanilamide was found most effective followed in order by sulfapyridine and sulfathiazole. (3) Recovery was apparently aided by the rapid production of antibodies (agglutinins).

13330

Failure of Sulfonamide Compounds in Treatment of Experimental *B. diphtheriae* (*Corynebacterium diphtheriae*) Infections of Guinea Pigs.

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To the best of my knowledge the effects of the sulfonamide compounds in the treatment of experimental *Corynebacterium diphtheriae* infections have not been reported upon and no reports are available upon their use in the treatment of diphtheria of human beings. As herewith reported, neoprontosil, sulfanilamide, sulfapyridine and sulfathiazole by oral administration to guinea pigs inoculated with 1 to 2 minimal lethal doses of the virulent bacillus have proven ineffective insofar as mortality is concerned although it is reasonable to assume that their administration to human cases of diphtheria may be helpful in combatting secondary infections with hemolytic streptococci, pneumococci and *Staphylococcus aureus.*

The virulence of the strain of *Corynebacterium diphtheriae* selected was such that subcutaneous injection (abdominal) of 0.6 to 0.8 cc of a suspension carrying about 1000 million per cc harvested in saline solution from 48-hour blood agar cultures, to guinea pigs weighing from 220 to 255 g proved lethal in about 4 days with the production of local lesions of edema and necrosis. Broth cultures were not employed in order to avoid the inoculation of preformed exotoxins.

Guinea pigs within the same age and weight range were then inoculated with 1 cc of the suspension (about 1000 million bacilli) by subcutaneous injection in the median abdominal line, the dose being from 1 to 2 minimal lethal doses in order to avoid overwhelmingly severe infections. The compounds were administered orally in dose of 0.05 g per animal corresponding to about 0.2 g per kg. The first dose was given immediately after inoculation with the second dose 4 hours later. Thereafter the compounds were given at 9 a.m. and 3 p.m. daily for 4 additional days corresponding to a total dosage of each compound of about 2 g per kg.

The results are summarized in Table I. Of 8 untreated controls 2, or 25%, survived, although showing local lesions of edema and necrosis. As shown in the table, none of the sulfonamide compounds employed were therapeutically effective insofar as survival was concerned. The degree of local edema with and without necrosis varied considerably among the treated and untreated animals so that no deductions on the therapeutic effects of the compounds on them are permissible. Cultures were made of the local lesions of 4 animals from each series, including the controls, on the second, fourth and seventh days after the institution of treatment and all were positive.

TABLE I.
Sulfonamide Compounds in Treatment of Experimental *Corynebacterium diphtheriæ* Infections of Guinea Pigs.*

Compounds†	No. animals	Survivals; Days 1	2	3	4	5	7	% survivals
Neoprontosil	8	8	8	6	2	1	1	12.5
Sulfanilamide	8	8	8	7	4	2	2	25
Sulfapyridine	8	8	7	6	3	2	2	25
Sulfathiazole	8	8	8	8	6	4	3	37.5
Controls	8	8	6	4	2	2	2	25

*Weights 220 to 255 g; inoculated subcutaneously with 1 cc of suspension carrying 1000 million per cc.

†Dose 0.05 g per animal orally; first dose given immediately after inoculation; second dose 4 hr later; thereafter 9 a.m. and 3 p.m. for 8 additional doses.

Summary. (1) Neoprontosil, sulfanilamide, sulfapyridine and sulfathiazole by oral administration proved ineffective in the treatment of experimental *Corynebacterium diphtheriae* infections of guinea pigs insofar as survival was concerned.

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Inhibitory Action of Amine-Oxidase and Tyrosinase upon the Vasoconstrictor Effect of Hypertensin.

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The publications of Holtz[1, 2] confirmed by Bing[3, 4] suggest that the vasoconstrictor principle which is found in the ischemic kidney is identical with hydroxytyramine. The nature of this pressor amine suggests that it may be destroyed enzymatically by means of two processes: by deamination of its side chain or by oxidation of its nucleus, since the latter possesses a phenolic character. Both Holtz and Bing favor the first mentioned view and regard the destruction as due to the action of amine-oxidase. Schroeder and Adams[5, 6] furnished experimental and clinical evidence showing that tyrosinase—a ferment which acts on the α-dihydrophenols and their corresponding α-quinones—decreases the blood pressure of hypertensive animals and inactivates angiotonin, tyramine and adrenalin *in vitro*.

The substance (hypertensin or angiotonin) which is formed through the interaction of renin and the pseudoglobulins of the serum, can readily be detected because of its direct vasoconstrictor action on Loewen Trendelenburg preparations of the posterior extremities of frogs.[7, 8] This test represents an adequate method for the investigation of hypertensin activity and thus may be used for the determination of any enzymatic action capable of destroying such pressor amines.

Our investigations revealed that hypertensin proves to be very labile if it is incubated at 20-39°C for a short time with extracts containing amine-oxidase. Blaschko[9, 10] reported that tissue extracts contain an amine-oxidase which destroys the vasoconstrictor proper-

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[1] Holtz, P., *Klin. Wschr.*, 1937, **16**, 1561.

[2] Holtz, P., und Heise, R., *Arch. Exp. Path. u. Pharm.*, 1939, **191**, 87.

[3] Bing, R. J., *Am. J. Physiol.*, 1941, **132**, 497.

[4] Bing, R. J., and Zucker, M. B., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 343.

[5] Schroeder, H. A., and Adams, M. H., *J. Exp. Med.*, 1941, **73**, 531.

[6] Schroeder, H. A., *Science*, 1941, **93**, 116.

[7] Houssay, B. A., et Taquini, A. C., *C. R. Soc. Biol.*, 1938, **128**, 1125.

[8] Garreton, A., Croxatto, R., Fuenzalida, O., e Viveros, R., *Rev. Arg. de Card.*, 1941, **8**, 1.

[9] Blaschko, H., and Richter, D., *J. Physiol.*, 1937, **90**, 1.

[10] Blaschko, H., and Schlossmann, H., *J. Physiol.*, 1940, **98**, 130.

ties of adrenalin and tyramine. Since this enzyme is present in appreciable quantities in the liver of certain cephalopodes,[11] we decided to study the action of an extract prepared from the liver of the squid, *Sepia officinalis.*

The recent work of Schroeder[5] induced us to examine, furthermore, whether the vasoconstrictor action of hypertensin—tested on the hind legs of the frog—is inhibited by tyrosinase. The results of these experiments are in agreement with those obtained following the use of amine-oxidase. Probably tyrosinase inactivates the vasoconstrictor substances because it leads to the oxidation of their phenolic nucleus while amine-oxidase would inactivate the same substances by deaminating the side chain.

Experimental Findings. Previous tests proved that if a mixture of renin obtained from hog kidney by the method of Helmer and Page[12] is incubated during 10-15 minutes with hypertensinogen ("renin activator") of cattle serum, purified by the method of Page and Helmer,[13] it assumes the ability to cause intense vasoconstriction in the hind limbs of the Chilean frog *(Caliptocephalus gayii)* perfused at constant pressure. 0.2 mg of renin and 0.5 ml of hypertensinogen thus incubated for 10-15 minutes suffice to reduce by 100% the number of drops leaving the abdominal vein. The same mixture incubated either before or after the 15-minute period with an extract of the liver of the squid within a very short time completely loses its vasoconstrictor ability, indeed in many cases it assumes vasodilator properties. This extract obtained according to the specifications of Blaschko[11] is rich in amine-oxidase. The dose of 0.05 ml of the liver extract completely destroys the vasoconstrictor effect of hypertensin within 5-10 minutes. A tyrosinase-containing purified extract of the mushroom *(Psalliota campestris)* obtained by the method of Nelson[14] likewise possesses the ability to destroy the activity of hypertensin following a short period of incubation (Table I).

We are now engaged in a purification of the two enzymes but meanwhile our first concern was to identify them. A comparative study of various tissues of the squid showed that the muscles possess a much less pronounced hypertensin-destroying ability than the liver. In agreement with the findings of Blaschko,[11] this difference may be ascribed to the comparatively small quantity of amine-

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[11] Blaschko, H., *J. Physiol.,* 1941, **99**, 364.

[12] Helmer, O. M., and Page, I. H., *J. Biol. Chem.,* 1939, **128**, 757.

[13] Page, I. H., and Helmer, O. M., *J. Exp. Med.,* 1940, **71**, 29.

[14] Ludwig, B. J., and Nelson, J. M., *J. Am. Chem. Soc.,* 1939, **61**, 2601.

TABLE I.

Effect of Amine-oxidase and Tyrosinase on Vasoconstrictor Potency of Hypertensin as Tested on the Hind Leg of the Frog.

Protocol No.	Substances added following 15 min. incubation, 36°C	Initial output No. of drops per min. prior to extr. administration	Max. output (maximal No. of drops per min. after extr. administration)	Min. during which max. effect evident
68	.1 ml renin + .5 ml hypertensinogen	40	0	3
81	.1 ml renin + .5 ml hypert. + .1 ml. extr. of squid	46	50	—
82	.1 ml ren. + .5 ml hypert. + .1 ml liver extr. of squid boiled 2 min.	50	3	5
85	.1 ml ren. + .5 ml hypert. + .1 ml liver extr. of squid + urea 3/M	36	15	3
92	.1 ml ren. + .5 ml hypert. + .1 ml liver extr. of squid + octyl alcohol	31	0	6
94	.1 ml ren. + .5 ml hypert. + .1 ml liver extr. of squid + 2.5 mg NaCN*	35	33	10
189	.1 ml ren. + .5 ml hypert.	28	1	4
169	.1 ml ren. + .5 ml hypert. + .25 ml tyrosinase	50	63	9
173	.1 ml ren. + .5 ml hypert. + .25 ml tyrosinase boiled 5 min.	65	0	8
194	.1 ml ren. + .5 ml hypert. + .25 ml tyrosinase + .01 g sodium benzoate	35	0	4
191	.1 ml ren. + .5 ml hypert. + .25 ml tyrosinase + .003 g NaCN*	57	0	3
200	.1 ml renin + .5 ml hypert. + .25 ml tyrosinase + .001 g NaCN*	70	12	4
197	.1 ml renin + .5 ml hypert. + .25 ml tyrosinase + octyl alcohol*	45	70	8

*The NaCN and the octyl alcohol acted on the respective ferments during several hours before these were mixed with renin and hypertensinogen.

In order to save space only one experiment of each series is reported in this table.

oxidase present in muscular tissue. Boiling destroys the action of the extracts. The presence of octyl alcohol destroys 60-100% of the antihypertensin activity. Urea in a 3 times molar concentration appreciably reduces this activity when kept in contact with the extract during 24 hours. On the other hand, HCN does not suppress the activity of the extract. This furnishes an indirect proof that the enzyme is an amine-oxidase.

The mushroom extract appears to be very sensitive to HCN and sodium benzoate, especially if these act on the enzyme for several hours. Both of these substances separately can completely or almost completely inhibit the activity of the enzyme. Octyl alcohol, on the other hand, has no appreciable effect. The activity of the mushroom extracts (some obtained by the method of Keilin and Mann[15]) was measured in the Warburg apparatus using catechol as substrate. The results ran parallel with those obtained in the perfused hind legs of the frog. The oxygen consumption was completely inhibited by HCN but not affected by octyl alcohol.

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[15] Keilin, D., and Mann, T., *Proc. Roy. Soc. SB.*, 1938, **125**, 186.

Discussion. The inhibitory action of amine-oxidase and tyrosinase on the product of the interaction between renin and hypertensinogen constitutes an indirect proof that the vasoconstrictor substance is a phenolic pressor amine as has been suggested by Holtz, Bing and Schroeder. Even though the enzymes used in these experiments have not been completely purified up to the present, their different behavior towards HCN, octyl alcohol, etc., is in agreement with the conception that the activity of squid extracts is due to amine-oxidase and that of the mushroom extracts to tyrosinase.

Summary. The vasoconstrictor substance (hypertensin, angiotonin) obtained by the interaction of renin and hypertensinogen and tested on the Loewen-Trendelenburg preparation is destroyed (enzymatic destruction) in contact with amine-oxidase (extract of *Sepia officinalis* liver) or tyrosinase (extract of *Psalliota campestris* liver).

13332

Sex and Proteinuria of Mice.*

L. F. Wicks. (Introduced by E. V. Cowdry.)

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While engaged in an investigation of possible systemic effects of methylcholanthrene on mice, many samples of their urine were analyzed. These urines frequently contained a surprisingly large amount of coagulable protein. This was regarded as due to the toxicity of the hydrocarbon until examination of normal, untreated and apparently healthy animals also revealed a high proteinuria. Further observations disclosed that this proteinuria is essentially sex-linked, for it was encountered very conspicuously in the males and usually only to a much smaller degree in females. The urine samples were from 9 pure strains (Swiss, A, New Buffalo, Old Buffalo, D, C_3H, CBA, AKA, C57 Black) and mixed stock mice. The mixed stock animals were of vague ancestry but definitely no

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* Aided by a grant from the U. S. Public Health Service.

TABLE I.

Proteinuria Differences in Groups of Male and Female Mice of Various Ages and Strains.

♂		♀	
Strain A.			
¾	++	1	tr.
1½	+	1¼	0
2	+++	1½	0
3	++++	1¾	f. tr.
3-4	+++	2-4	tr. to +
4	near. ++++	3-4	tr.
5½	+++	4	f. tr.
6	++++	4¾	tr.
*6+	+++	*6+	f. tr.
6+ d.g.	+++	10	tr.
8	+++	†10	+
10	++++	19 (1 mouse)	0
Old Buffalo.			
¾-1	+ to ++	1	tr.
1¼	+	1¼	f. tr.
1¾	+++ to ++++	1¾	0
1-2	+++	1¾ d.g.	tr.
2	++	1-2	++
2½	+++	*2-3	f. tr.
2, 3	++ to +++	3-4	+
3	+++ to ++++	7-8	tr.
3½	near. +++	8-10	+ to ++
4	+++	10-12	+
4,5	++ to +++		
8 (1 mouse)	+++		
AKA.			
9-10	+++	9-10	0
Same		Same	
9½-10½	+++ or more	9½-10½	0
CBA.			
4-5	++++	3-4	++
Same		Same	
4½-5½	++	3½-4½	0
5½-6½	++++	4-5	less than +
Mixed Stock.			
1¼ K-C	+++	1	0
1¼	+	1¼	+ or less
1½ K-C	near. +++	1½ K-C	tr. to +
1¾ K-C	++++	3-3½	f. tr.
1¼ K-C, d.g.	+++	3½-4¼	tr.
2 K-C	++		
3	+++	Same group diff. mice	+
3-3¼	++++	6	tr.
3-4	+++	7	+ or less
4	++++	Indefinite	f. tr.
Several (?)	+++	*Several (?)	0
Indefinite	+++		
6+	+		
Indefinite	near. +++		

New Buffalo

♂		♀	
1	+ to ++	1	tr.
1½	tr.	1 d.g.	tr.
2	++++	2	+ or more
2½	+++	2 d.g.	tr.
2½ d.g.	+++	2½	++
2¾	++++	3¼	tr.
3	+++ to ++++	5	tr.
3¼	+++	6½	0
5	++++	7	tr.
7	++++	*8	0
*8	++ to +++		
		Swiss.	
¾-1	++ to +++	¾-1	0
1½-2	near. ++	2¼	tr.
3	++ to +++	2-3	tr.
4	++++	4	tr.
4 d.g.	++++	4 d.g.	tr. to +
4 d.g.	+++	5	0
5	+++	6+	near. ++
6	++++	7	tr.
7	+++ or more	8	tr. to +
7-8	+++ or more	9-12	tr.
*10-12	+++		
		D.	
3	+++ to ++++	3-4	++
Same 3½	+++	Same 4-5	+
6	++ to +++	6	tr.
10	++ to +++	10	f. tr.
15	near. ++++	15	tr.
		C57 Black.	
2-4	++	2-4	0
4-5	near. ++++	4-5	++ or over
Same 5-6	++++	Same 5-6	near. +++
2-3	++	2-3	f. tr.
Same 3-4	++++	Same 3-4	tr.
Repeat	++++	Over 12	tr.
		C$_3$H.	
5½-6	+++	5-6	++
Repeat	+++	Repeat	near. +++
9	++++	2nd repeat	++ to +++
Repeat	+++	9-10	tr. or over
9-9½	+++ or over	Repeat	++
Repeat	+++	2nd repeat	near. ++
		12	tr. to +
		Repeat	++
		2nd repeat	near. ++

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*Unusually large volume.
†On high protein diet for 8 months.
0—No test with T.C.A.
f. tr.—Faint trace, just visible turbidity.
tr.—Trace, cloud only, no ppt.
d.g.—Different group of animals.

longer of pure line. (Those designated "K-C" were a cross between "mixed stock" and a hairless strain.) The C_3H mice exhibited less sex difference and more irregularity than any other strain examined. The results were otherwise quite generally consistent throughout, proteinuria being detected to considerable extent in males from 3 weeks of age onward, and in greatly reduced intensity in females, even when quite old.

The urine was collected in specially constructed cages, remotely similar to the pattern of Levine and Smith[1] for rats. In each, a double screen bottom covered a wax-lined funnel which led into a test tube containing a little toluol. Provision was made so that the drinking water could not drip into the funnel.

Since mice do not readily tolerate fasting, it was necessary to supply them with food while in the collection cages. Besides, fasting mice show a great reduction in urine volume. The food supplied in the collection cages was a mixture of washed casein, starch, salt mixture, cod liver oil and lard, a diet the mice consumed readily. The last named ingredients were in sufficient quantity to form a stiff paste which the animals could not scatter easily. The preparation was also contained in "non-scatter" food cups.[2] Both food and feces were frequently extracted and tested, but neither ever exhibited more than a trace of soluble, coagulable protein. The mice received this unbalanced diet for not more than 24 hours at one time.

The urine samples as represented in Table I were the pooled excretions of 2 to 4 similar animals, except in the case of very young ones where 5 were used. The left columns give the approximate age in months of each group of mice and the right columns the degree of proteinuria. The urines were centrifuged rapidly, and 1 cc of clear fluid was mixed with 5 cc of 15% trichloracetic acid. In these preliminary observations, the relative amounts of precipitate were arbitrarily recorded as "trace" to "four plus". This designation is considerably higher that that in clinical usage. A quantitative method has been devised and numerical data will be reported subsequently along with a description of the procedure.

Upon investigating the chemical nature of the urinary protein, we were surprised to find that while traces of albumin and globulin are present, the greater portion seems to be nucleoprotein. No reference has been met in the literature on the excretion of large amounts of such a protein in the urine of mice, either as a normal or abnormal occurrence. There is a series of observations by one worker, Parfentjev,[3, 4, 5] on the presence of a protein in the urine of normal male mice. He believed it to be a chondromucoid substance and adhered to this view when he later isolated the material in collaboration with Perlzweig.[5] Our analyses indicate that it is a nucleoprotein rather than a mucoprotein. Cold acidification of the urine with acetic acid causes a slow separation of the substance which may then be redissolved in very dilute alkali and reprecipitated. The solutions resulting from many acid hydrolyses of the protein failed to give any reaction for reducing sugar, although the Molisch carbohydrate test was always positive, and hydrochloric acid hydrolysis yielded no evidence of sulphate. Kiliani's desoxy-carbohydrate reaction was obtained as well as the crude aniline acetate test for pentose. Phosphate was always found, and the ammoniacal silver nitrate test for purines proved positive. More complete details may be reported elsewhere.

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[1] Levine, H., and Smith, A. H., *J. Lab. and Clin. Med.*, 1925, **11**, 168.

[2] Hawk, P. E., and Bergeim, O., *Practical Physiol. Chem.*, 1937, 11th Edition, p. 909.

To exclude the possibility that this urinary protein is of extrarenal origin, and where found in males might be due to contamination with spermatic fluid, urine was drawn from the bladder of mice killed immediately before collection. This revealed a protein content as high as that of voided urine.

The consideration that the urinary protein seems a nucleoprotein makes it unlikely that its presence in urine is due to a renal defect. There are, however, two observations recorded in the literature which made it necessary to test this possibility. Gorer[6] has described extensive degenerative lesions in the kidneys of very old mice of both sexes belonging to A, C57 and CBA strains. Preliminary histological examinations of the kidneys of our younger mice revealed only occasional minor lesions. Griffith and Wade[7, 8] have shown that on certain diets poor in choline, degenerative kidney changes may develop in rats. While our diet was not likely to be so deficient, experiments were carried out in which a daily supplement of 0.5 mg of choline hydrochloride was injected for a period of 3 months. This supplement did not diminish the excretion of urinary protein. The excretion was, however, found to be affected by the protein content of the diet. One containing 33% of casein

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[3] Parfentjev, I. A., Proc. Soc. Exp. Biol. and Med., 1932, **29**, 1285.

[4] Parfentjev, I. A., *Ibid.*, 1933, **30**, 1064.

[5] Parfentjev, I. A., and Perlzweig, W. A., *J. Biol. Chem.*, 1933, **100**, 551.

[6] Gorer, P. A., *J. Path. and Bact.*, 1940, **50**, 25.

[7] Griffith, W. H., and Wade, N. J., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 188.

[8] Griffith, W. H., and Wade, N. J., *Ibid.*, 1939, **41**, 333.

increased the proteinuria, while a diet with only 5% casein diminished it markedly.

Since the proteinuria in mice is associated with sex, the effect of castrating young male mice was studied. The degree of proteinuria remained unchanged for many weeks following the operation, but was greatly decreased after about 3 months. Addis[9] has reported a similar result for albuminuria of male rats. Bell[10] noticed the appearance of albuminuria as young male rats reached maturity. Our rats show no excretion of protein, and we have found very little mention of any marked proteinuria of these animals.

In connection with the apparent sex-linked character of the proteinuria of mice, the recent report of Crabtree[11] is of great interest. She observed differences in structure of Bowman's capsule between most male and female mice. She cites the experiments of Selye,[12] who noticed that treatment of female mice with testosterone propionate may change the parietal lamina of Bowman's capsule.

Summary. A protein with chemical characteristics of a nucleoprotein was found to be excreted in large amounts by male mice of various ages and strains. Female mice excrete much less protein, frequently none at all. A table is appended.

For the castration of animals and the examination of sections, I am much indebted to Dr. William Cramer.

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Prolongation of Estrus by Injection of Suspensions of Estrogen Crystals.

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The influence of the method of administration upon the duration of the effect of estrogenic compounds has early been recognized and described by several authors.[1] D'Amour and Gustavson[2] showed that 3.8 γ estrone in aqueous solution can produce estrus in rats when the dose is given in a single injection, while only 0.74 γ is necessary if multiple injections are administered. A solution in oil, however, does not show significant differences if administered in one or fractional doses. This is in agreement with the findings of Behrens and collaborators[3] that the spreading of the M.E.D. of an aqueous solution of estrone resulted in prolongation of estrus. Parkes[4] showed that even massive doses of estrone produced only a transitory feminization of the plumage of Brown Leghorn capons. Similarly, Miescher and collaborators[5] found that estrus in rats after subcutaneous injection of as much as 50 γ estrone in oil lasted not more than 5 days. Deansly and Parkes[6] implanted crystals of 2.0 to 3.2 mg estrone intramuscularly in Brown Leghorn capons and found that the feminization of the plumage lasted for approximately 3 months. Recently, it has been shown that the implantation of estrogen crystals or pellets is highly efficient in therapeutics.

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[9] Addis, T., *Proc. California Acad. Med.*, 1931-32, 38, and personal communications.

[10] Bell, M. E., *J. Physiol.* (Brit.), 1933, **79**, 191.

[11] Crabtree, C., *Science*, 1940, **91**, 299.

[12] Selye, H., *J. Urol.*, 1939, **42**, 637.

Experimental. Obviously, the implantation of estrogen crystals is not a simple procedure. The administration of a suspension of estrogens, as for instance, 5 mg estrone in 1 cc of an aqueous solution of 5.2% gum acacia, is a practical modification of the implantation method. The vehicle is promptly resorbed and leaves the finely divided crystals in the tissues. The crystals are small enough to pass through a 26 gauge hypodermic needle. The suspended estrone settles very slowly and a suspension can be obtained by shaking the mixture before injection. A suspension of 5 mg estrone in 1 cc sesame oil was also prepared. Due to the solubility of the estrone in the oil, approximately 1 mg is present in solution and the rest in suspension. Furthermore, an aqueous suspension was made from estradiol, containing 5 mg in 1 cc of 5.3% gum acacia solution. Microscopical examination of the suspensions showed a very satisfactory uniformity regarding the size of the crystals.

Castrated female rats and mice, as they are employed for assay work of estrogens, were used. Rats received one single injection

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[1] Allen, E., Hisaw, F. L., and Gardener, W. W., *Sex and Internal Secretion,* E. Allen, Baltimore, 1939.

[2] D'Amour, F. E., and Gustavson, R. G., *J. Pharm. and Exp. Therap.,* 1936, **57**, 472.

[3] Behrens, B., Heubner, W., Koll, W., and Kulz, F., *Arch. Exp. Path. und Pharm.,* 1937, **186**, 121.

[4] Parkes, A. S., *Biochem. J.,* 1937, **31**, 579.

[5] Miescher, K., Scholz, C., and Tscopp, E., *Biochem. J.,* 1938, **32**, 141.

[6] Deansly, R., and Parkes, A. E., *Proc. Royal Soc., London,* 1937, **124**, 279.

TABLE I.
Duration of Estrus in Rats and Mice Following Injection of Estrogens.

Series	No. of animals	Dose, mg	Vol., cc	Duration of estrus (days)		Drug used
				Range	Avg	
				Rats.		
I	9	.5	.1	5-20	15.5	Estrone, aqueous suspension
II	10	.5	.1	6-24	15.0	” ” ”
III	10	.5	.1	11-39	23.1	” ” ”
IV	9	.5	.1	13-41	23.5	” ” ”
V	10	.5	.1	9-19	14.2	Estradiol, ” ”
VI	9	.5	.1	5-20	9.6	Estrone, oil suspension
VII	9	.5	.4	2.0	2.0	” solution in oil
				Mice.		
I	10	.25	.05	10-26	18.0	Estrone, aqueous suspension
II	10	.15	.10	13-40	20.8	” ” ”
III	10	.15	.10	9-21	15.3	” ” ”
IV	10	.15	.10	7-20	10.6	Estradiol ” ”
V	6	.25	.20	4-10	6.0	Estrone, solution in oil

of 0.1 cc subcutaneously, amounting to 0.5 mg of the estrogens in the form of suspensions as illustrated in Table I. As a control, 0.5 mg estrone dissolved in 0.4 cc oil was given to Group VII. Ninety percent of the animals in all groups came into estrus within 24 hours, the rest within 48 hours.

Duration of estrus was followed by vaginal smears in each animal and the average for each group was determined. The estrus in the rats injected with a solution of estrone in oil (Group VII) lasted only 2 days, while the effect was very much prolonged in all groups receiving an estrogen in a form of a suspension both in oil and in water. There was a great variation in the duration among the different animals within the groups. However, the averages show a significant prolongation of effects with the aqueous suspension of estrone in the Groups I-IV and with estradiol in Group V.

The estrus obtained with the estrone suspension in oil was definitely shorter in duration than that with estrone in water. Probably the dissolved estrone is removed from the oil in the tissues and the suspended estrone dissolves again in the remaining oil, thus hastening the absorption as compared with the aqueous suspension.

The experiments in mice essentially corroborate the findings in rats. Doses of 0.25 and 0.15 mg were used. While the duration of estrus following the injection of a solution of 0.25 mg of the estrone in oil is longer than that resulting from the injection of 0.5 mg in oil in rats, the aqueous suspension at both dose levels used in mice results in a prolongation of estrus of the same order as observed in rats. The injection of estradiol in an aqueous suspension produced also some prolongation of the duration of estrus. A number of rats and mice were sacrificed during the later period of the estrus and the uteri examined histologically. They presented hypertrophy of the muscular layer and submucosa. The changes were more pronounced in the rats and in these the effect was more marked in the animals injected with the aqueous suspension than with the suspension in oil.

Inspection of the places of injection when the aqueous suspension was used showed no irritation, nor were deposits visible. In addition to the convenience of administration, the freedom of oil eliminates the possibility of reactions as they occasionally occur in patients sensitive to oil. Clinical investigation in humans, using the same aqueous suspension of estrone, corroborates the results in experimental animals.[7]

Conclusions. The duration of estrus in castrated rats and mice was measured after injection of equal amounts of estrone as an aqueous suspension, as a suspension in oil, and as a solution in oil. The suspensions, particularly the aqueous ones, by far outlasted the effect of the solution. The aqueous suspension of estradiol was also followed by a prolonged stage of estrus. This method offers a convenient way to produce prolonged effects with free estrogens without resorting to surgical implantation of crystals.

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Effect of Vitamin C Deficiency on Action of Different Types of Barbiturates.

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Generally, the liver is assumed to be of importance for the destruction of the so-called short-acting barbiturates, but not for the long-acting group. Recent work by Scheifley and Higgins[1] using partial hepatectomy, and by us[2] employing chemical liver damage, brought the unexpecteed result that the liver does not seem to be equally important for the destruction of pentothal sodium, a widely used ultra-short-acting intravenous anesthetic. Simultaneously, we found that the type of liver damage appeared to be of importance, as there was little difference in the action of the various types of barbiturates if severe fatty infiltration of the liver in rats was produced by dietary means.

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[7] Freed, S. C., *J. Clinic. Endocrin.*, in press.

[1] Scheifley, C. H., and Higgins, G. M., *Am. J. Med. Sci.*, 1940, **200**, 264.

[2] Richards, R. K., and Appel, M., *Anesthesia and Analgesia*, 1941, **20**, 66.

In pursuance of the possibilities of nutritional factors in the detoxification of barbiturates, we studied the rôle of vitamin C in this connection.

While it is well known that this vitamin affords a certain protection against some poisons, most of the recent publications concern the influence of drugs upon the vitamin C excretion. Here, the barbiturates were found to increase vitamin C output in the urine (Ritz, Samuels, and Addiss,[3] Svirbely,[4] Longenecker, Fricke and King.[5]

Our approach was to study the effect of vitamin C depletion upon the action of different barbiturates. Barbital sodium was used as a long-acting drug, pentobarbital sodium (Nembutal) as a short-acting hypnotic, and the ultra-short-acting pentothal sodium, on account of its different chemical structure, as a thiobarbiturate, and for the reasons mentioned before. These compounds were administered intraperitoneally in groups of guinea pigs and their sleeping time observed. This was repeated after 7 to 10 days, and then the animals were placed on a vitamin C-free diet on which they remained up to 33 days.

During this period the injection of the barbiturate was repeated at intervals and the sleeping time noticed. (See Table I—Barbital Sodium.)

Four groups of 9 to 13 animals were studied with barbital sodium. The average sleeping times did not show a consistent or significant increase even after prolonged vitamin C depletion which was fatal for some animals.

The next table shows similar experiments with Nembutal. (See Table II—Nembutal.) Here, the difference between the normal and C-depleted animals was striking. One week of C-free diet was without influence (Group A), but after 2 weeks in some groups (B and D), later in others (C), the sleeping time is markedly prolonged. Injections of 50 mg vitamin C subcutaneously daily for 3 to 4 days with (Group C) or without (Group D) replacement of normal diet promptly restored normal sleeping times.

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[3] Ritz, N. D., Samuels, G. T., and Addiss, G., *J. Pharm. and Exp. Ther.*, 1940, **70**, 362.

[4] Svirbely, J. G., *Abstracts Meet. Am. Chem. Soc.*, Oct., 1938.

[5] Longenecker, H. E., Fricke, H. H., and King, C. G., *J. Biol. Chem.*, 1940, **135**, 497.

TABLE I.
Barbital Sodium Intraperitoneally in Guinea Pigs.

			Sleeping time						
			Avg		Range				
Group	No. of animals	Days between tests	hr	min	hr	min	hr	min	Diet and remarks
A	10		3	38	3	14 -	3	56	Normal
	10	7	2	48	1	50 -	4	15	”
	10	7	4	35	3	49 -	5	49	C-free 7 days
	10	7	2	55	2	19 -	4	04	” 14 ”
B	13		2	52	1	05 -	4	48	Normal
	13	8	3	42	1	58 -	5	15	”
	12	14	3	51	1	39 -	5	47	C-free 14 days
	10	10	4	00	2	18 -	6	10	” 24 ”
	9	7	4	15	1	55 -	5	33	” 31 ”
C	9		5	10	1	36 -	6	42	Normal
	10	7	4	06	2	15 -	5	18	”
	10	20	6	09	5	09 -	8	39	C-free 14 days
	9	8	5	30	5	04 -	7	02	” 22 ”
D	10		5	15	3	35 -	6	26	Normal
	9*	7	4	47	3	01 -	7	27	”
	10	28	5	51	4	14 -	7	57	C-free 23 days

*One animal failed to sleep.
Groups A and B received 100 mg/kg. Groups C and D, 130 mg/kg.

The significance of the differences in the sleeping time occurring on normal and on C-free diet was proven statistically on Group A and Group B. In Group A, the longest sleeping time on a normal diet, 4 hours, 57 minutes, was compared with the shortest one on a C-free diet, 7 hours, 14 minutes. The standard error ϵ for the normal experiment was 10 minutes and for the C-free one 32 minutes. The significance of the difference was calculated by the formula, $\frac{M_1 - M_2}{\sqrt{\epsilon_1^2 + \epsilon_2^2}}$, where M_1 and M_2 are the averages of the two groups. The value found is 4.0 which makes the result statistically highly significant.

A similar calculation was performed in Group B where the normal sleeping time of 4 hours, 47 minutes was compared with 6 hours, 29 minutes on the C-free diet. Here, also, the highly significant value of 9.4 was obtained.

Since the differences in Groups D and E are practically identical to those in Groups B and C, no calculations were performed for Groups D and E.

Addition of 5 mg of vitamin C to a normal diet in non-depleted guinea pigs (Group E) did not exert a significant influence.

The following series comprises the experiments with pentothal sodium. (See Table III—Pentothal Sodium.) The results are

TABLE II.
Nembutal (30 mg/kg) Intraperitoneally in Guinea Pigs.

			Sleeping time							
			Avg		Range					
Group	No. of animals	Days between tests	hr	min	hr	min		hr	min	Diet and remarks
A	10		4	57	4	00	-	5	45	Normal
	10	7	4	01	3	40	-	6	08	’’
	10	7	4	20	2	39	-	5	26	C-free 7 days
	8	13	7	14	5	08	-	8	37	’’ 20 ’’
	8	7	7	44	5	51	-	9	20	’’ 27 ’’
B	11		3	53	2	44	-	5	58	Normal
	11	8	4	47	3	58	-	5	49	’’
	11	15	6	55	6	21	-	7	09	C-free 14 days
	10	10	6	29	5	55	-	7	03	’’ 24 ’’
	5	7	6	26	5	41	-	6	50	’’ 31 ’’
C	10		3	25	2	44	-	4	23	Normal
	10	8	3	52	2	24	-	5	25	’’
	8	30	7	13	5	40	-	8	36	C-free 24 days
	4	8	4	49	4	33	-	5	12	Normal diet 8 days; 50 mg ascorbic acid daily 3 days
D	12		4	38	3	05	-	6	13	Normal
	12	8	4	33	3	09	-	6	16	’’
	12	14	7	06	6	44	-	7	23	C-free 14 days
	9	10	4	16	3	39	-	7	17	’’ 24 ’’ ; 50 mg ascorbic acid daily last 4 days
	9	7	4	58	3	54	-	6	21	Normal diet last 7 days
E	10		4	27	2	49	-	5	25	Normal
	10	11	4	20	2	52	-	5	10	’’
	10	7	3	30	2	14	-	4	41	’’ ; 5 mg ascorbic acid daily last 5 days
	9	16	3	48	3	27	-	5	34	Normal

similar to those with barbital, since vitamin C-depletion does not markedly influence the sleeping time. In Groups D and E the animals were first tested during C-depletion and addition of ascorbic acid did not change the effect in Group D, and only slightly shortened it in Group E. We had the impression that, in general, animals subjected to repeated pentothal injections were less resistant against vitamin C-deficiency than those on barbital and nembutal. Animals showing extreme loss of weight or inability to walk as a result of progressive vitamin C-depletion, were not included in these experiments.

Since it is known that vitamin C depletion causes glycogen deficiency in the liver (Altenburger[6]), we investigated whether or not the low glycogen may be responsible for the prolonged sleeping time. Therefore, experiments were conducted with all 3 barbiturates in guinea pigs after 36 hours' starving. With all 3 drugs sleep

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[6] Altenburger, E., *Klin. Wschnschr.*, 1936, **15**, 1129.

TABLE III.
Pentothal Intraperitoneally in Guinea Pigs.

Group	No. of animals	Interval between tests, days	Sleeping time							Diet and remarks
			Avg		Range					
			hr	min	hr	min		hr	min	
A	10		6	08	5	41	-	6	40	Normal
(male)	10	10	5	10	4	18	-	5	35	”
	8	33	4	08	2	01	-	9	31	C-free 24 days, 6 animals died within following week
B	10		4	06	1	31	-	5	40	Normal
(male)	5	28	5	05	3	46	-	6	10	C-free 28 days
C	12		3	41	1	18	-	4	41	Normal
(male)	12	22	5	11	2	41	-	7	00	”
	11	9	4	45	3	03	-	6	04	C-free 9 days
D	8		2	53	1	26	-	4	48	C-free 2 weeks
(male)	8	7	3	38	3	12	-	5	29	” 3 ”
	8	6	3	09	2	21	-	3	29	Normal + 50 mg ascorbic acid daily last 5 days
E	12		4	42	2	17	-	6	58	C-free 32 days
(female)	10	7	4	07	2	57	-	6	33	” 39 ”
	10	4	2	44	1	54	-	3	47	Normal + 50 mg ascorbic acid daily 4 days
	10	6	3	24	1	16	-	4	47	Normal

Group A received 30 mg/kg. Group B, 45 mg/kg. Groups C, D, E, 35 mg/kg.

lasted, as an average, one hour longer than in unstarved animals, which is not a very marked change of the effect. A certain prolongation of sleep in starved rabbits has also been observed by Blackberg and Hrubets[7] in their experiments with pentothal. Inanition as a reason for the prolonged sleeping time can also be excluded. We observed a marked prolongation of sleeping time in the nembutal experiments at a time when the C-depletion had not yet progressed sufficiently to cause a loss of weight, while, on the other hand, the loss of 100-150 g in similar experiments with barbital was not followed by prolongation of sleep. In this connection the work of Spellberg and Keeton[8, 9] is also of importance inasmuch as they found marked fatty changes in the liver of guinea pigs after vitamin C-depletion. We have, therefore, conducted numerous fat determinations in the liver of guinea pigs in different stages of vitamin C-depletion by means of Soxhlet extraction. Normal guinea pigs were found to have a liver fat

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[7] Blackberg, S. N., and Hrubetz, C., *J. Lab. and Clin. Med.*, 1937, **22**, 1224.

[8] Spellberg, M. A., and Keeton, R. W., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 570.

[9] Spellberg, M. A., and Keeton, R. W., *Am. J. Med. Sci.*, 1940, **200**, 688.

TABLE IV
Relationship Between Sleeping Time and Liver Fat in Guinea Pigs.
(25 mg/kg nembutal given intraperitoneally.)

Days on C-free diet	% fat in liver	% glycogen in liver	Sleeping time		Remarks
			hr	min	
21	6.8	—	8	04	
28	6.6	0.21	7	32	
28	9.6	0.93	7	32	
28	13.2	—	7	09	
28	9.1	—	9		
28	6.2	—	9		
23	5.4	1.33	2	02	100 mg Vit. C last 3 days before nembutal
23	24.7	0.42	2	12	
23	5.1	2.81	2	26	
23	8.0	2.02	2	37	
33	8.3	—	4	43	
33	10.8	—	4	17	
33	4.0	—	3	12	100 mg Vit. C last 4 days before nembutal
33	15.1	—	4	04	
33	3.9	—	3	11	
Normal diet	16.2	—	3	35	Starved 36 hrs

content of 2.6 to 4.2%. In our animals, fat accumulation in the liver was not as pronounced or as regular as in the experiments of Spellberg and Keeton. Values of 5.0 to 24.7% were found by us in guinea pigs after 3 to 4 weeks of C-free diet. (See Table IV.)

Table IV shows the relation between diet, fat content of the liver, glycogen and sleeping time after nembutal injection, and the influence of vitamin C administration. As can be seen, there was no consistent increase in fat content with prolonged C-depletion and, similarly, there was no significant correlation between the fat content and the sleeping time. The latter showed a marked increase in C-depleted animals as compared with normal sleeping times (Table II), while the fat content was frequently only little greater than in animals on a normal diet. Spellberg and Keeton[9] claim that addition of vitamin C to the C-free diet did not prevent the development of the fatty livers which they attribute to other factors in the diet. Table IV illustrates also that the addition of vitamin C was not regularly effective in restoring normal fat content, but, and this is significant, it restored normal sleeping time irrespective of the fat content. The lack of connection between sleeping time and liver fat is also illustrated in an animal starved for 36 hours which had a normal sleeping time in spite of a liver fat content of 16.2%.

Discussion. The results of our experiments demonstrate that only a certain type of barbiturate seems to be influenced in its duration of effect by vitamin C-deficiency. This group is the so-called short acting barbiturate, of which nembutal is a representative, and we have no reason to assume that the other members of this group would behave differently. Since sufficient evidence has been brought forward that the liver destroys this type of barbiturates, one has, necessarily, to consider the possibility of liver damage as an explanation for the prolonged duration of action in the vitamin C-depleted animals. As we have demonstrated in this work, the appearance of fat due to the deficient diet or the low glycogen content is obviously not responsible. Neither were we able to find histological changes in the liver such as can be demonstrated after liver damage produced by drugs, as chloroform or carbon tetrachloride. We have, therefore, reason to believe that the vitamin C may be directly, or indirectly, connected with the destruction of these types of barbiturates. King[10] has recently mentioned the decrease in certain liver enzymes in the state of vitamin C-deficiency. Thus, it may be possible that the failure of liver enzymes accounts for the slower destruction of these drugs. Further support for this viewpoint is given by the fact that barbital is not significantly influenced in its duration of action by vitamin C-deficiency. This substance is known not to be destroyed in the body, but is excreted to a large extent unchanged by the kidney.

In addition, our experiments with vitamin C avitaminosis demonstrate further that pentothal does not behave as other short-acting barbiturates, and thus substantiates evidence brought forward by other authors, as well as ourselves, that this drug is not destroyed in the liver.

Summary. Vitamin C deficiency does not significantly alter the sleeping time of guinea pigs with either barbital or pentothal. The sleeping time with nembutal is markedly prolonged. This prolongation is not due to decreased liver glycogen, inanition, or increased liver fat, and can be promptly brought to normal by addition of vitamin C. We believe, therefore, that this vitamin must be connected in one way or another with the metabolism of nembutal and similar barbiturates. Furthermore, these experiments give additional evidence that the metabolism of pentothal is essentially different from that of nembutal and its related barbiturates.

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[10] King, C. G., Biochem. Soc. Meet., Chicago, April, 1941.

13335

Bis-para-aminobenzoyl-l-cystine.

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Abel and Geiling[1] concluded unstable sulfur was an essential part of the insulin molecule. Du Vigneaud[2] stated insulin probably was a derivative of cystine and later it was reported[3] that while the disulfide linkage *per se* was not responsible it was nevertheless necessary for the insulin action. The importance of sulfur in the insulin molecule has prompted many investigators to test a number of relatively simple miscellaneous organic sulfur compounds, sulfur-containing guanidine derivatives, certain cystinyl peptides and cystine itself for hypoglycemic activity.[4] None of the compounds investigated exhibits or approximates the physiological behavior of insulin.[4]

The present experiments were carried out to test a cystine derivative, bis-para-aminobenzoyl-l-cystine,* for possible hypoglycemic properties.

These studies† were made on rats fasted 15-18 hours. After amputation of a thin segment of the tail, blood was milked into an oxalated glass capsule and blood sugar determined by the Shaffer-Hartman Somogyi method[5] on filtrates prepared by the zinc hydroxide precipitation procedure of Somogyi.[6] Blood was withdrawn before subcutaneous administration of the sodium salt of the cystine compound and ½, 1, 2, 3, and 5 hours thereafter. In Fig. 1 are shown the results of a typical experiment and it is to be noted that blood sugar values for the animals receiving the cystine compound closely approximate those obtained for the control group, whereas the rats receiving 1.5 units of insulin per kg show a well defined hypoglycemia. Almost identical results were obtained in

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[1] Abel, J. J., and Geiling, E. M. K., *J. Pharm. and Exp. Therap.*, 1925, **25**, 423.

[2] du Vigneaud, V., *J. Biol. Chem.*, 1927, **75**, 393.

[3] du Vigneaud, V., Fitch, A., Pekarek, E., and Lockwood, W. W., *J. Biol. Chem.*, 1931, **94**, 233.

[4] Braun, C. E., Mason, M. B., and Brown, C. L., *J. Chem. Educ.*, 1938, **15**, 261.

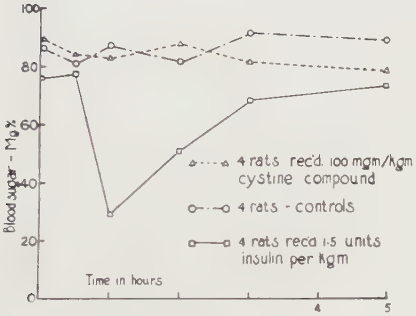
* This compound was made available to us through the courtesy of Dr. R. L. Shriner of the Department of Organic Chemistry, University of Illinois.

† This investigation was supported in part by the D. J. McCarthy Foundation and by the Smith, Kline and French Laboratories Fellowship Fund.

[5] Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, 1933, **100**, 695.

[6] Somogyi, M., *J. Biol. Chem.*, 1930, **86**, 655.

20 other rats using doses of from 50-200 mg/kg of the cystine compound. In Fig. 2 are shown the results obtained in 9 Wistar rats after injection of 3.5 g/kg of glucose (10% solution) intraperitoneally and it is to be noted that the glucose tolerance curve of the rats receiving the cystine compound is similar in all respects to that of the control animals.



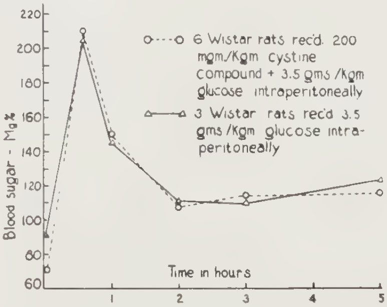


Fig. 1.

Fig. 2.

Fig. 1. Comparative effectiveness of bis-p-aminobenzoyl-l-cystine and insulin on blood sugar of rats.

Fig. 2. Glucose tolerance studies on Wistar rats.

Summary. Bis-p-aminobenzoyl-l-cystine was found to be devoid of hypoglycemic properties in normal rats. This compound also did not affect the glucose tolerance curves of Wistar rats.

13336 P

Increase in Blood Lipids of Fasted Mice.

P. L. MacLachlan. (Introduced by E. J. Van Liere.)

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The observations of Entenman, Changus, Gibbs and Chaikoff[1] on adult dogs fail to confirm the claim that fasting produces a lipemia. Since fasting mice promptly show a marked degree of fatty infiltration into the liver[2] it was considered of interest to determine whether a lipemia accompanies fasting in this species.

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[1] Entenman, C., Changus, G. W., Gibbs, G. E., and Chaikoff, I. L., *J. Biol. Chem.*, 1940, **134**, 59.

[2] Hodge, H. C., MacLachlan, P. L., Bloor, W. R., Stoneburg, C. A., Oleson, M. C., and Whitehead, R., *J. Biol. Chem.*, 1941, **139**, 897.

TABLE I.
Data on Blood Lipids of Fasted Mice.

No. of mice	Body wt loss, %	Phospholipid, mg %	Neutral fat cholesterol, mg %	Total lipid, mg %
		Normals.		
13	—	256 (200-286)*	275 (203-384)	531 (403-637)
		1 day fasted.		
10	16	323 (302-365) (+26)†	291 (271-328) (+6)	614 (573-661) (+16)
		2 days fasted.		
10	21	349 (291-442) (+36)	301 (231-402) (+9)	651 (525-844) (+23)
		3 days fasted.		
5	25	321 (237-393) (+25)	418 (322-575) (+52)	739 (559-968) (+39)

*Figures represent range of values.
†Figures represent percentage changes from normal.

Three-months-old, male, albino mice, previously maintained on a diet of oats and Purina Dog Chow were fasted (water *ad libitum)* in individual wire cages so that coprophagy was minimal, for 1-, 2-, and 3-day periods. Blood samples were drawn by heart puncture and the whole blood analyzed by standard methods for phospholipid[3] and acetone soluble lipid (neutral fat and cholesterol).[4]

The increasing loss in body weight as fasting progressed is in agreement with earlier findings.[2] Of particular interest is the rise in the phospholipid content of the blood as a result of fasting; maximum values were found on the second day of starvation. This increase in blood phospholipid can be explained on the assumption that phospholipid serves as a transport mechanism in the intermediate metabolism of fat. It is also noteworthy that there is a gradual rise in the total blood lipid content as fasting progressed. This is mostly accounted for, on the first and second days of fasting, by the increased phospholipid values. However, on the third day of fasting, although the phospholipid decreased slightly, the average total lipid value reached its maximum, due in part to very high values for acetone soluble lipid (>500 mg %) for 2 of 5 animals. Further work is in progress.

Summary. On fasting, 3-months-old, male, albino mice show increased blood phospholipid and total lipid values.

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[3] Bloor, W. R., *J. Biol. Chem.*, 1929, **82**, 273.

[4] Bloor, W. R., *J. Biol. Chem.*, 1928, **77**, 53.

13337 P

Effect of Ingestion of Various Pyridine Compounds on Excretion of Specific Fluorescent Substances in Urine.*

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In previous publications[1, 2] attention has been called to 2 fluorescent substances in the urine, the concentrations of which appear to depend upon the body stores of nicotinic acid. The composition of these 2 substances which we have designated F_1 and F_2 is as yet unknown. They are characterized by their fluorescence with ultraviolet light, F_1 having an emission spectrum ranging from 4,000 to 4,800 angstrom units with a maximum at 4,350 Å, and F_2 having an emission spectrum ranging from 4,200 to 5,400 Å with a maximum at 4,550 Å. With suitable extraction procedures the excretion of these 2 substances can be measured quantitatively with a fluophotometer. We have found that F_1 is normally present in relatively small quantity in the urine but increases markedly in pellagra. F_2, on the other hand, is present in greater quantity under normal conditions but disappears in pellagra. The administration of nicotinic acid to a pellagrin causes a prompt reduction of F_1 and the reappearance of F_2. Similarly, the administration of nicotinic acid to a normal individual causes F_2 to increase well beyond the normal limits. To explain these facts, we have suggested the hypothesis that F_1 is converted into F_2 through the agency of some nicotinic acid containing enzyme. In nicotinic acid deficiency, failure of such conversion causes F_1 to accumulate and F_2 to disappear, whereas in the presence of this catalyst, F_2 is formed in quantities which bear some relation to the supply of nicotinic acid.

It seemed of interest to ascertain the effect of various pyridine and pyrazine compounds on the excretion of F_2 in human subjects in order to determine if there were any parallelism between this property and the ability to cure pellagra. The compounds chosen for testing were: nicotinic acid, nicotinamide, sodium nicotinate, dinicotinic acid, trigonelline, 2-6 dimethyl nicotinic acid, quinolinic acid, pyrazine monocarboxylic acid, pyrazine, 2,3-dicarboxylic acid, pyridine β-carboxylic acid diethyl amide (coramine) and sulfapyridine. These

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* Aided by a grant from Mead Johnson and Company, Evansville, Indiana.

[1] Najjar, V. A., and Wood, R. W., Proc. Soc. Exp. Biol. and Med., 1940, **44**, 386.

[2] Najjar, V. A., and Holt, L. E., Jr., *Science*, 1941, **93**, 20.

TABLE I.
Excretion of F_2 in Urine (Najjar-Wood Units) During a 4-hour Period after Ingestion of Various Pyridine and Pyrazine Compounds.

Compound ingested	Dose, mg	Before treatment	After treatment
Nicotinic acid	100	6	60
Nicotinamide	100	12	87
Pyridine β-carboxylic acid diethylamide (coramine)	2500	10	104
Trigonelline	200	15	11
2-6,dimethyl dinicotinic acid K salt	200	10	13
Quinolinic acid	200	4	5
Pyrazine monocarboxylic acid	100	7	2
Pyrazine 2,3-dicarboxylic acid	200	5	4
Dinicotinic acid	200	8	9

compounds were fed to normal male adults, the urine being followed for a period of several hours before and after the test dose. With one exception 3 experiments were carried out with each compound. The analytical procedure used for the determination of F_2 was that described by Najjar and Wood.[1]

Results. It was found that only 4 of the compounds tested: namely, nicotinic acid, nicotinamide, sodium nicotinate and coramine produced a rise in the excretion of F_2 in the urine. The rise was significant in each of the experiments and was of comparable magnitude. With the other compounds no appreciable increase in excretion of F_2 occurred, even with doses many times that required to cause an increase in F_2 with nicotinic acid. Typical protocols obtained in one subject are shown in Table I.

In a few experiments we tested the effect of sodium nicotinate given intravenously. This procedure resulted in an increase of F_2 comparable to that obtained by oral ingestion.

Comment. It is worthy of note that the ability of these pyridine compounds to produce an increased excretion of F_2 in the urine shows a marked parallelism to the efficacy of the compound in the treatment of human pellagra. In the experience of Sydenstricker[3] all 4 of the compounds which in our hands gave rise to increased F_2 excretion have been clinically effective, whereas of the compounds which gave negative results in our tests only one (quinolinic acid) was found by him to be antipellagric for man and that in doses well in excess of those customarily employed. It is possible that if we had used large doses of some of these compounds, as we did in the case of coramine, our results might have been different.[†]

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[3] Sydenstricker, V. P., personal communication.

[†] We wish to express our indebtedness to Drs. G. S. Palmer, E. Meyer and F. Zak who assisted in these studies, and also to Dr. C. E. Bills of Mead Johnson and Company, who provided us with a number of the compounds tested.

13338

The Pantothenic Acid Content of Royal Jelly.*

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Considerable effort has been made to explain the physiological factors responsible for the development of one female larva of the honey-bee *(Apis mellifera L.)* into a worker while another becomes a queen. The queens and workers are produced from identical larvae, as it is possible to rear queens from any female larvae less than 3 days old by providing them with the proper food. For the first 2 days after hatching all female larvae receive a diet of royal jelly, a secretion of the pharyngeal glands of the workers.[1] During the third day the diet of the larvae that are to become workers is changed while the queen caste continues to receive royal jelly. This difference in diet appears to be responsible for the morphological and functional differences between the two castes.

Royal jelly has been assayed for various vitamins with the idea that its phenomenal properties may be accounted for by the presence of liberal amounts of one or more of these growth factors. Experiments were conducted by Hill and Burdett[2] which lead them to conclude that the worker bees add vitamin E to the royal jelly but withhold it from the food of the worker larvae. It has since been shown[3-6] that there are only negligible amounts of vitamin E present in royal jelly. According to Melampy and Jones[7] royal jelly contains no demonstrable amounts of vitamins A and C. Using the rat curative method Haydak and Palmer[8] found that royal jelly contains approximately 9 μg of thiamin per g of dry material. The phe-

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* Published with the approval of the Director of the Texas Agricultural Experiment Station as technical contribution No. 691.

[1] Snodgrass, R. E., *Anatomy and Physiology of the Honeybee*, New York, McGraw-Hill Book Co., 1925, p. 174.

[2] Hill, L., and Burdett, E. F., *Nature*, 1932, **130**, 540.

[3] Schoorl, P., *Z. Vitaminforsch.*, 1936, **5**, 246.

[4] Mason, K. E., and Melampy, R. M., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 459.

[5] Evans, H. M., Emerson, G. A., and Eckert, J. E., *J. Econ. Ent.*, 1937, **30**, 642.

[6] Haydak, M. H., and Palmer, L. S., *J. Econ. Ent.*, 1938, **31**, 576.

[7] Melampy, R. M., and Jones, D. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 382.

[8] Haydak, M. H., and Palmer, L. S., *J. Econ. Ent.*, 1940, **33**, 396.

nomenal properties of royal jelly in developing queen bees can hardly be attributed to vitamins in it that have heretofore been studied.

The rat assay methods for the determination of vitamins in royal jelly leaves much to be desired. The quantity of royal jelly obtainable is sufficient for the use of only a limited number of animals. For most assays it is necessary to pool the royal jelly from several colonies to have sufficient for the assay. These difficulties are obviated by microbiological methods of assay.

The universal distribution of pantothenic acid in animal tissues and its biological importance which has been reviewed by Williams[9] prompted us to study the pantothenic acid content of royal jelly. Assays for pantothenic acid were made on fresh royal jelly by the microbiological method.[10] The moisture content was determined on each sample of royal jelly so as to permit expressing the pantothenic acid on both the fresh and dry basis. The samples of royal jelly from the same colony were secured at different times and from different cells after the colony had been dequeened.

From the data in Table I it is seen that royal jelly contains an average of 183 μg of pantothenic acid per g on a fresh basis and 511 μg per g on a dry basis. This is considerably higher than for any other known material. Yeast and liver are 2 of the richest known sources of pantothenic acid. According to Jukes[11] dried brewers' yeast contains an average of 200 μg and liver 180 μg per g. On this basis the pantothenic acid content of royal jelly is more than 2.5 times that of yeast. Using the microbiological assay, Strong,

TABLE I.
Pantothenic Acid Content of Royal Jelly.

				Pantothenic acid	
Sample No.	Colony No.	Moisture, %	Dry matter, %	Fresh, μg/g	Dry, μg/g
1	I	63.8	36.2	142	392
2	I	67.5	32.5	140	431
3	I	61.1	38.9	147	378
4	II	62.1	37.9	215	567
5	II	62.8	37.2	230	618
6	III	66.5	33.5	205	612
7	III	65.5	34.5	200	580
	Avg	64.2	35.8	183	511

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[9] Williams, R. J., *Biol. Rev.*, 1941, **16**, 49.

[10] Pennington, D., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 1940, **135**, 213.

[11] Jukes, T. H., *J. Nutrition*, 1941, **21**, 193.

et al.,[12] reported substantially lower values for yeast and liver than those found by Jukes with chick assay method. Thus, when the comparison is made with values obtained by the microbiological method the ratio of pantothenic acid in royal jelly to that of yeast is approximately 6 to 1.

Of the vitamins studied in royal jelly pantothenic acid is the only one in which it is unusually rich. Whether or not pantothenic acid is in any way responsible for the development of female larvae into queen bees remains to be studied. The presence of pantothenic acid in such relatively large amounts does suggest that it might be one of the factors responsible for the development into queens of the female larvae which continue to receive royal jelly.

Summary. It has been found that royal jelly is the richest known source of pantothenic acid. On a dry basis it contains an average of 511 μg per g which is between 2.5 and 6 times as much pantothenic acid as is present in yeast or liver.

13339 P

Influence of Age on Survival of Respiration, Spinal Reflexes, Pupillary Responses and Heart Action.

W. A. Selle.

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Despite the many observations reported on survival of tissues and organs, little information is available concerning the influence of age on survival time. Several observers[1-5] have reported that young animals are more resistant to anoxia and asphyxia than are adults, and Selle and Witten[6] found that the primitive respiratory mechanism

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[12] Strong, F. M., Feeney, R. F., and Earle, Ann, *Ind. Eng. Chem., Anal. Ed.*, 1941, **13**, 566.

[1] Reiss, M., and Haurowitz, F., *Klin. Wchnschr.*, 1929, **8**, 743.

[2] Avery, R. C., and Johlin, J. M., Proc. Soc. Exp. Biol. and Med., 1932, **29**, 1184.

[3] Kabat, H., and Dennis, C., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 534.

[4] Selle, W. A., and Witten, T. A., *Proc. Am. Physiol. Soc.*, 1941, **53**, 253.

[5] Himwich, N. E., Alexander, F. A. D., and Fazekas, J. F., *Proc. Am. Physiol. Soc.*, 1941, **53**, 193.

[6] Selle, W. A., and Witten, T. A., Proc. Soc. Exp. Biol. and Med., 1941, **47**, 495.

(gasping) itself survives much longer in young animals than in old. Thus, the head of a new-born rat or dog gasps 40 to 80 times during a period of 30 to 60 minutes following ligation of the cerebral vessels or decapitation; the head of a half-grown or adult animal ceases gasping within a few seconds.

The present studies were undertaken to determine whether the anoxic survivals of other physiological processes, such as pupillary responses, trunk reflexes and heart action, parallel that of the respiratory mechanism.

Survival of the gasping mechanism was determined as described previously.[6] Maximal dilatation of the pupil was taken to be a satisfactory indication of cessation of pupillary activity. Following the onset of anoxia the pupils constrict to pin point size. Later they dilate and for a time only respond to bright light. The duration of trunk reflexes (movement of tail or foot) was determined by the application of painful induction shocks to cutaneous areas of the abdomen and foot. To follow cardiac beats, the heart was exposed by a midline incision of the chest within 2 minutes after spinal transection or after thoracic respiration had ceased.

Results on 3 series of animals are given in the accompanying table.

TABLE I.
Age Factor in Survival of Respiration, Pupillary Responses, Spinal Reflexes and Heart Beat.

Age, days	No. animals	Gasping ceased, sec.	Max. dilation of pupil, sec.	Trunk reflexes abolished, sec.	Heart beat ceased	
					Ventr., min.	Aur., min.
Rats						
Premature	6	1885	—	1530	111	146
1	15	1765	—	1215	88	103
3	17	1667	—	835	85	96
4	12	1420	—	628	85	102
7	10	970	256	460	90	99
14	14	305	140	122	72	78
21	10	121	82	67	28	36
42	10	45	52	43	12	17
100	10	15	47	37	13	16
Rabbits						
1	10	1465		1320	103	113
3	9	1280		1080	72	84
7	6	452		252	51	58
14	3	67		38	15	18
90	2	15		20	14	19
Dogs						
1	3	2100		920	47	58
3	2	1652		840	38	42
7	3	530		420	25	31
14	3	352		270	8	11
35	2	94		74	6	8
65	1	35		45	7	9

The remarkable survival of the respiratory mechanism in young animals is obviously not unique; other physiological processes survive similarly.

In early age groups the survival of trunk reflexes is about two-thirds that of gasping; in the older age groups there is no clear difference. The survival of pupillary responses in the 7-day-old rat is about 3 times that of the adult. Heart action for all species studied is approximately 7 times longer in 1-day-old animals than in adults.

13340

Body Size and Metabolism of Liver Slices *in vitro*.

Max Kleiber.

*From the College of Agriculture, University of California, Davis.**

Grafe, Reinwein and Singer[1] concluded from micro respiration trials that the metabolic rate of animal tissues *in vivo* was restricted by a central regulator to a fraction of the rate of oxidation of the tissues *in vitro*. The summated *in vitro* metabolism[†] of the rat according to Grafe is twice as great as the metabolic rate *in vivo* and the tissues of a steer respire *in vitro* 20 times as fast as they do in the living animal. Based on similar work Terroine and Roche[2] derived their rule: The metabolic rate *in vitro* per unit weight of homologous tissues of large and small animals is the same even though the metabolic rate per unit weight of the tissues in the living animals decreases systematically with increasing body size.

In contrast to Grafe's conclusion, Field, Belding and Martin[3] demonstrated that the summated metabolic rate of rat tissues *in vitro* amounted to 89% of the basal metabolic rate of the living rats. Since the ratio of *in vitro* to *in vivo* metabolic rate according to

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[*] The author acknowledges the help of several colleagues at Davis, especially his assistant, Arthur H. Smith.

[†] Summated *in vitro* metabolism = metabolic rate per unit weight *in vitro* multiplied by the weight of each tissue in the living animal and summated over all its tissues.

[1] Grafe, E., Reinwein, H., and Singer, *Biochem. Z.*, 1925, **165**, 102.

[2] Terroine, E. F., and Roche, J., *Compt. Rend. Acad. Sci.*, 1925, **180**, 225.

[3] Field, J., 2nd, Belding, H. S., and Martin, A. W., *J. Cell. Comp. Physiol.*, 1939, **143**, 14.

Grafe should be greater the larger the animal it is desirable to test his theory on larger animals. Martin has started such a test on dogs.[4]

The experiments reported in this paper were designed to test whether or not the metabolic rate *in vitro* of homologous tissues of different animals is related to the body size of these animals (Terroine's rule).

Method. Livers were secured from 7 rats killed after 12 hours' fast, 9 rabbits killed aftter 24 hours' fast, 5 sheep fasted 48 hours and 2 sheep fasted 14 hours before killing. The liver of one horse and one cow killed after 12 hours' fast are included in this investigation, though the data thus obtained are not included in the calculation of the final result. All the animals were mature.

The oxygen consumption of the hand cut liver slices was measured in Warburg micro respiration apparatuses using as a medium a balanced ion solution with glucose and phosphate buffered at pH 7.4.[5] The liver slices were in the apparatus 15 to 30 minutes after the animal was killed. The readings were started 10 to 15 minutes after the installation of the manometers in the bath, which was maintained at a temperature of 37.0°C. The mean respiratory rate during the first half hour was used for the calculation of the results. The amounts of tissue used for the trials were measured by dry matter and micro-kjeldahl determinations. In some cases the N content alone was measured and the dry matter was calculated using $\frac{\text{dry matter}}{\text{N}}$ ratios that were determined in extra liver samples. These ratios ranged from 7 to 9. The results could thus be expressed in cubic millimeters of oxygen consumed per hour per milligram of dry tissue. (Q_{O_2}).

Results. Column 5 of Table I shows that the Q_{O_2} of the liver slices *in vitro* decreases systematically with increasing size of the animal. The only exception to this rule is the result on the cow

TABLE I.
Oxidation Rate of Liver Slices *in Vitro.*

Animal	No. of animals	No. of measurements of Q_{O_2}	Mean body wt, kg	Mean Q_{O_2}, mm^3 O_2 per mg dry tissue per hr
Rats	7	20	.245	7.25 ± 0.43
Rabbits	9	54	3.3	4.17 ± 0.17
Sheep	7	38	18	2.54 ± 0.18
Horse	1	4	182	2.1
Cow	1	3	410	2.6

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[4] Martin, A. W., personal communication.

[5] Kleiber, M., and Cole, H. H., *Am. J. Physiol.*, 1939, **125**, 747.

whose liver slices respired with the same intensity as those of the sheep. This latter result would be in agreement with Terroine's rule; it is, however, not conclusive since it was obtained from a single cow that was lactating and had fasted only for 12 hours.

The difference between the Q_{O_2} of the rat and rabbit liver slices and also that between the rabbit and sheep slices are statistically highly significant. In order to derive a quantitative relation between the metabolic rate of liver slices *in vitro* and the body weight of the animals the logarithm of Q_{O_2} is plotted against the logarithm of body weight as shown in Fig. 1. The Q_{O_2}'s as function of body weights of rats, rabbits and sheep can be represented by a straight line—calculated by the method of least squares—which is summarized by the equation—

$$\log Q_{O_2} = 0.721 - 0.24 \log W$$

or

$$Q_{O_2} = 5.26\ W^{0.24}$$

where Q_{O_2} = mm^3 oxygen consumed per mg dry tissue per hour
W = body weight of animal in kilograms.

The metabolic rate per unit weight of tissue is thus inversely proportional to $W^{0.24}$ which is practically the same as the fourth root of body weight (see dotted line in Fig. 1). This is the same function

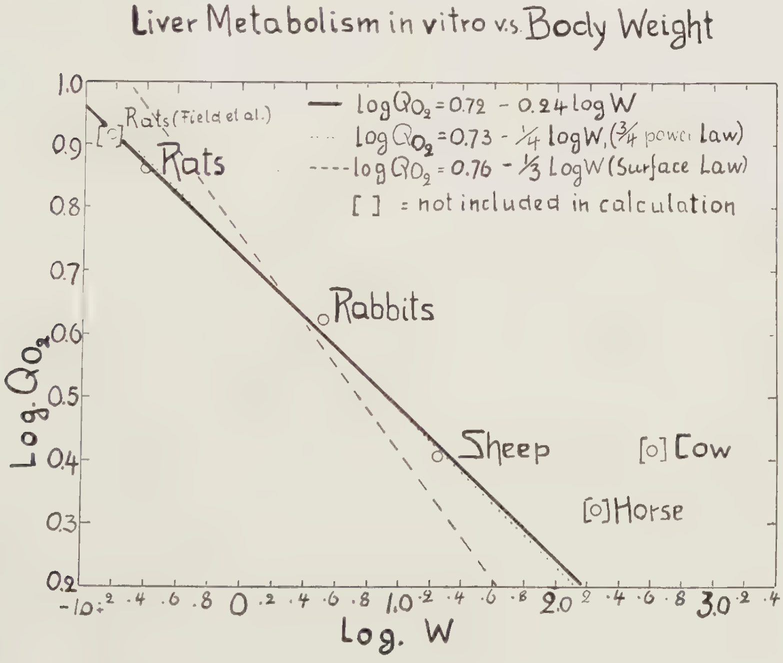

FIG. 1.

of body weight to which the basal metabolism per unit weight of warm blooded animals from rat to steer is most closely related. The metabolic rate of these animals is proportional to the ¾ power of their body weight.[6] The metabolic rate per unit weight is therefore proportional to $W^{3/4}/W$ or to $W^{-1/4}$. Fig. 1 shows that tissue metabolism follows the ¾ power rule more closely than the surface law which is indicated by the broken line. More measurements on cows and horses are necessary in order to answer the question whether or not the metabolism of their tissues *in vitro* deviates significantly from the rule which is valid for liver slices of rats, rabbits, and sheep. Fig. 1 indicates that the results of Field, Belding and Martin[3] on rats smaller than those used in our investigation fit very closely the function of body size and *in vitro* metabolism derived from our data.

Discussion. The results of this investigation indicate either that the metabolism of an animal is essentially determined peripherally by a condition of its tissues—often called, somewhat mysteriously, their oxygen requirement or energy requirement—or that the factors by which a central regulator (such as the nervous or the endocrine system) controls the metabolic rate of the tissues *in vivo* are still effective *in vitro,* a conclusion reached in earlier work on growth hormone rats.[5] Oxygen supply does not seem to be such a determining factor since the respiratory rates of tissue slices *in vitro,* with an equal abundance of oxygen for all, differ so greatly. The same reasoning rules out glucose or phosphate supply as major regulating factors. It can be assumed that a chemical or physical condition such as concentration of respiratory enzymes or the structural relation of cell components becomes established in the cell under the influence of a central regulator or one may assume that only cells with a metabolic condition that makes them fit into the body as a whole can survive in an intact organism and that these cells then retain this metabolic condition even when they are cut out of the organism and therefore retain a similar metabolic rate *in vitro* as they had *in vivo.*

Summary. The metabolic rates of liver slices from rats, rabbits, sheep, one horse and one cow have been measured *in vitro.* These metabolic rates per unit tissue weight for rat, rabbit and sheep liver decrease systematically and significantly with increasing body size. The metabolic rate per unit tissue weight is inversely proportional to the 4th root of body weight. Body size affects thus the metabolic rate *in vitro* in the same way as it affects the metabolic rate of the intact animal and the factors which determine the metabolic level *in vivo* seem still to be present in the surviving tissues cut out of the organism.

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[6] Kleiber, M., *Hilgardia,* 1932, **6**, 315.

13341 P

Effect of *Triturus* Toxin on Respiration of Rat Tissues *in vitro.*

Frederick A. Fuhrman and John Field, 2d.
Department of Physiology, Stanford University.

Twitty and coworkers[1] have shown that a very active toxin is present in the yolk of *Triturus* ovarian eggs and embryos. Some of the chemical properties and physiological actions of this substance have been described by Horsburgh, Tatum and Hall.[2] Among other actions the toxin paralyzes motor and sensory nerve endings and evokes central respiratory paralysis in the anesthetized cat.

Since it appeared possible that the effects on nervous tissue might be attributable, in part at least, to depression of oxidative metabolism, we have investigated the effects of graded concentrations of the toxin on the respiration of rat brain (cerebral cortex) slices. For comparison, similar experiments were carried out on kidney cortex and liver slices from the same animals. Methods of preparation of tissue slices differed somewhat from those used previously[3] and will be described elsewhere. Oxygen consumption was measured in cmm, N.P.T., per mg wet weight per hour, by the Warburg manometric method. A relatively pure preparation of the toxin was kindly furnished us by Drs. van Wagtendonk and Tatum.

Dried powder containing the toxin was taken up in Ringer's-phosphate-glucose[3] and brought to pH 7.2. The final concentrations in the respirometer vessels ranged from 0.43 mouse units (M.U.) per ml[2] to 850 M.U. per ml. Eight adult male rats were used, and at least 2 determinations were made at each dosage level. The lowest concentration was about 8 times the dosage required to kill a 20 g mouse in 10 minutes,[2] assuming uniform distribution in the body. The corresponding concentrations of pure toxin cannot be stated exactly, but there is evidence[4] suggesting that it ranged from about 0.006 to 1.3 mg per ml. The dried powder containing the toxin was free from proteins, peptids, fats, basic amino acids and alkaloids. Reducing sugar, minerals and some dialysable nitrogenous compounds, including neutral and acidic amino acids were present.[4]

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[1] Twitty, V. C., and Elliott, H. A., *J. Exp. Zool.*, 1934, **68**, 247; Twitty, V. C., and Johnson, H. H., *Science*, 1934, **80**, 78; Twitty, V. C., *J. Exp. Zool.*, 1937, **76**, 67.

[2] Horsburgh, D. B., Tatum, E. L., and Hall, V. E., *J. Pharm. and Exp. Therap.*, 1940, **68**, 284.

[3] Crismon, J. M., and Field, John, 2d, *Am. J. Physiol.*, 1940, **130**, 231.

It was found that the respiration of brain and kidney slices was affected only by the highest concentration used (850 M.U. per ml), while the respiration of liver slices was stimulated slightly with 85 M.U. per ml. These results are summarized in Table I, where it is shown that when any difference did appear between the control and experimental rates of oxygen consumption it was rather small and transient.

TABLE I.

Comparison of Respiratory Rates of Rat Brain, Kidney and Liver Slices in the Presence of High Concentrations of *Triturus* Toxin with That of Controls (Arbitrarily Taken as 100). Time Indicated is After Start of Run.

	850 M.U.		85 M.U.		"Inactivated"*	
Tissue	30 min.	120 min.	30 min.	120 min.	30 min.	120 min.
Brain	111	104	99	96	118	115
Kidney	134	107	100	97	108	84
Liver	174	113	119	128	108	60

*850 M.U. reduced to 100 M.U. by treatment with alkali. Brought to pH 7.2 before use.

It is not clear that this effect could be attributed to the toxin. Concentrations of 85 and 850 M.U. involved the presence of 1.33 to 13.3 mg of organic matter respectively, which might have served as additional substrate. When a solution of 850 M.U. per ml concentration was partially inactivated,[4] so that the strength was reduced to 100 M.U. per ml, there was no initial stimulation, but the respiratory rate decreased below the control levels after 120 minutes. This appears to indicate that more complex changes than simple inactivation of toxin occurred, and that the inactivated preparation was not an adequate control for testing the hypothesis that substrate rather than toxin was responsible for the initial rise in respiration with high doses.

In all cases pH measurements were made with a glass electrode before and after the runs, and little change in pH occurred. Frequent tests were made on mice to determine whether loss in toxicity occurred during the Warburg runs. This was not the case.

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[4] Tatum, E. L., and van Wagtendonk, W. G., unpublished.

Work on the effect of this toxin on glycolysis is now in progress.

Conclusions. Low concentrations of *Triturus* toxin had no effect on the respiratory rate of rat brain, kidney and liver slices *in vitro.* In the presence of 85 M.U. per ml in the case of liver, and of 850 M.U. per ml in the case of kidney and brain slices, slight transient stimulation of respiration occurred, but it is not clear that this could be attributed to the action of the toxin.

13342

Application of "Falling Drop" Measurement of Density to Quantitative Determination of Antibody in Immunological Reactions.*

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From the Hygienic Laboratory, University of Michigan, Ann Arbor, Michigan.

The serological methods for measuring antibody concentration are based on secondary reactions which are indirect in nature. The determination of the amount of protein precipitated by a specific antigen, a method first described by Wu and associates,[1] and since developed by Heidelberger and Kendall,[2] estimates directly the amount of antibody protein contained in a solution. The only serious disadvantage of this method is the time required.

It occurred to us that the rapid "falling drop" measurement of density for ascertaining protein concentration, as developed by Barbour and Hamilton,[3] might be applied to the quantitative determination of antibody. The technic of this test has been adequately described, and no essential modification has been made by the authors. The most troublesome source of error was maintaining a sufficiently constant temperature in the water bath.

The antiserums used in these experiments were freed of any suspended particles by centrifugation and the supernatant fluids used. The antigen, Type I pneumococcus polysaccharide prepared accord-

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* These studies were aided by a grant from the Horace H. Rackham School of Graduate Studies.

[1] Wu, PROC. SOC. EXP. BIOL. AND MED., 1928, **25**, 853; 1929, **26**, 737.

[2] Heidelberger and Kendall, *J. Exp. Med.*, 1929, **50**, 809.

[3] Barbour and Hamilton, *J. Biol. Chem.*, 1926, **69**, 625.

TABLE I.
Comparison of Falling Drop Rate of Antiserum and Precipitable Nitrogen, Following Reaction of Antiserum with Varying Amounts of Antigen.

Antiserum ml	Antigen, mg	Precipitable nitrogen, in mg*	Falling drop rate,† sec. per 20 cm	Ratio of falling drop rate to precipitable N
1.0	.00	.00	34.2	
1.0	.05	.28	36.6	127
1.0	.10	.32	38.6	121
1.0	.15	.33	39.4	120
1.0	.20	.33	39.3	120
1.0	.25	.33	39.4	120

*Estimated by difference.

†Average of 5 determinations on supernatant fluids after precipitates were removed by centrifugation.

ing to the method of Heidelberger, Kendall and Scherp,[4] was dissolved in 0.85% saline. Dilutions in which 1.0 ml contained from 0 to 0.25 mg of polysaccharide were all adjusted to the same density by the addition of solid sodium chloride. Equal amounts of the antigen dilutions and antibody were mixed, and allowed to stand overnight in the icebox; the precipitates were removed by centrifugation and the supernatant fluids tested for nitrogen by the Kjeldahl method as modified by Pregle.[5] The precipitable nitrogen was deter-

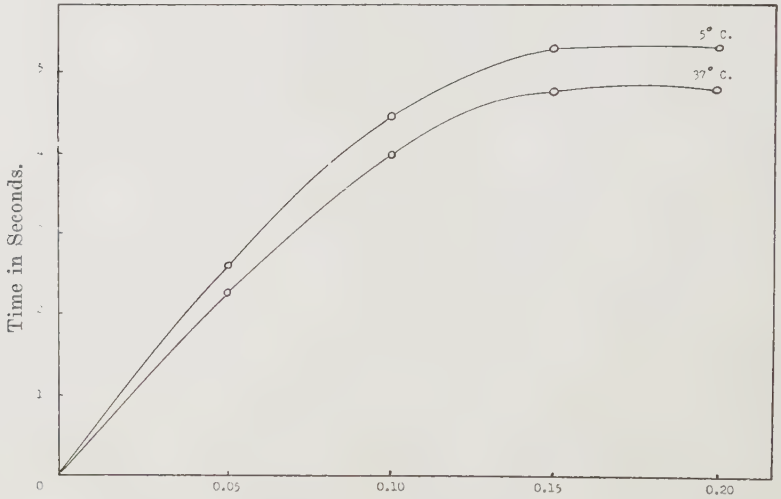

CHART 1.
Effect of Temperature of Incubation on Extent of Antigen-antibody Reaction, as Measured by Falling Drop Method.

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[4] Heidelberger, Kendall and Scherp, *J. Exp. Med.*, 1936, **64**, 559.

[5] Pregle, *Quantitative Organic Micro Analysis, Philadelphia,* P. Blakiston's Son and Co., 1934.

mined by difference. Simultaneously, falling drop measurements were made to determine the densities of the supernatant fluids. The results of a typical experiment are given in Table I. The last column of this table shows the ratio of falling drop time to precipitable nitrogen. The constancy of this value indicates a close parallel between the data obtained by the two methods.

In the course of these studies, it was noted that the temperature at which the antigen-antibody system was incubated influenced the reaction as measured by the falling drop method. This is in accordance with Heidelberger's findings,[6] and the results are plotted in Chart 1.

Summary. The falling drop method devised by Barbour and Hamilton for the determination of the concentration of protein solutions has been found applicable to the estimation of the antibody content of antipneumococcus serum. After the precipitate has been separated from the reacting mixture by centrifugation, the density of the supernatant fluid can be determined in less than one minute by this method.

13343

A Silver-glass Micro-electrode for Use in a Micromanipulator.

George P. Fulton. (Introduced by F. H. Pratt.)

From the Department of Biology, Boston University, Boston, Mass.

In an investigation of the neuro-motor mechanism of small blood vessels,[1] the author needed to make micro-electrodes for the stimulation of small nerve fibers. The non-polarizable type (micro-salt-bridge), described by Ettisch and Péterfi,[2] Taylor,[3] Gelfan,[4] and Field,[5] was not necessary. Various polarizable types described in the literature were found to be inadequate. The platinum-quartz micro-electrode, devised by Taylor[3] and used by Pratt and Reid[6] and by Steiman[7] for the study of single muscle fibers, proved to be expensive, required an oxygen flame to draw, and broke easily. The micro-electrode described by Sen[8] may be produced in quantity by the deposition of metallic platinum, gold, or silver on the surface of quartz or glass micro-needles, using Brashear's method for silvering glass or the method of sputtering with a high voltage discharge in a vacuum. Electrodes of this type had to be insulated individually by the application of shellac with a small brush. It was found that the insulation did not resist a strong faradic current.

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[6] Heidelberger, *Bact. Rev.*, 1939, **3**, 41.

[1] Fulton, G. P., and Lutz, B. R., *Science*, 1940, **92**, 223.

[2] Ettisch, G., and Péterfi, T., *Pflüger's Archives*, 1925, **208**, 454.

[3] Taylor, C. V., Proc. Soc. Exp. Biol. and Med., 1925, **23**, 147.

[4] Gelfan, S., Proc. Soc. Exp. Biol. and Med., 1926, **23**, 308.

[5] Field, M. E., *Skand. Arch. f. Physiol.*, 1935, **72**, 175.

[6] Pratt, F. H., and Reid, M. A., *Science*, 1930, **72**, 431.

A 3-inch length of silver suture wire (No. 28 or No. 30) was inserted in a closely-fitting, soft glass capillary and drawn in a minute gas flame to produce an insulated silver-glass micro-electrode. The type of micro-burner described by Chambers and Kopac[9] was used. When a satisfactory tip was obtained, the end of the silver wire was fused to a piece of copper wire. The copper wire and the initial portion of the shank of the electrode were then sealed in a 5-inch length of glass tubing (6 mm in diameter), by means of a beeswax-resin cement. This tube constituted the electrode holder and was inserted in an Emerson micromanipulator in place of the holder supplied with the instrument.

Micro-electrodes of one to 5 micra in diameter at the tip were obtained by this method. If the silver core was sealed by glass at the tip, the insulation could be removed from this point by passing a strong induced current through the electrode. The period of service was prolonged by cleaning the electrode with a small camel-hair brush beneath the surface of the electrolyte in which it had been used.

For use in stimulation the silver-glass micro-electrode was connected to an induction coil and made the active terminal for unipolar stimulation. It was polarizable, and the minute bubble of hydrogen gas, which appeared at the tip when the current was made and increased in magnitude during the time of stimulation, served to indicate the moment and duration of the stimulus. This property of the polarizable electrode has been of particular value in motion picture recording of the responses of small blood vessels to stimulation of minute nerves in the field under the microscope.

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[7] Steiman, S. E., *Am. J. Physiol.*, 1937, **118**, 492.

[8] Sen, B., PROC. SOC. EXP. BIOL. AND MED., 1930, **27**, 310.

[9] Chambers, R., and Kopac, M. J., *McClung's Handbook of Microscopical Technique*, New York, 2nd Edition, 1937.

13344 P

Effect of Bilateral Splanchnicectomy upon Renal Blood Flow in Hypertension.

Arthur Selzer and Meyer Friedman.

From the Harold Brunn Institute for Cardio-Vascular Research, Mount Zion Hospital, San Francisco, Calif.

It has been suggested by Peet, Woods and Braden[1] that the frequently successful results observed by them in hypertensive patients following splanchnicectomy might have been caused by a decrease in the tonus of the renal arterioles, thus permitting a better renal blood flow. In this communication we are reporting the renal blood flow and glomerular filtration rate as measured by the diodrast and inulin clearances, respectively, in two hypertensive patients before and after splanchnicectomy.

Description of Cases. Case No. I. L. G., male, age 32. History of severe headaches and difficulty in vision of 6 months' duration. On physical examination, the patient was found to have a blood pressure of 220/135 mm Hg, and severe retinal changes consisting of marked tortuosity and beading of the smaller arteries, many areas of exudate and hemorrhage with marked arterio-venous impression. The urine examination revealed occasional hyaline casts and erythrocytes. The specific gravity of a concentrated urine specimen (12 hours) was 1.020. Intravenous pyelography revealed no abnormalities. The diodrast clearance and inulin clearance were performed on July 26, 1940. On October 6, 1940, the patient was operated upon by Dr. Howard Naffziger at the University of California Hospital. At that time, a bilateral resection of the greater splanchnic and lesser splanchnic nerves together with the lower part of the sympathetic chain was performed. The patient withstood the procedure well despite an immediate and severe drop in blood pressure to a shock level immediately following the operation. After several days, the blood pressure again began to rise and on October 16th, the day on which the diodrast and inulin clearances were again performed, the blood pressure was 250/150 mm Hg.

Case No. II. F. P., male, age 52. History of severe headaches and marked impairment of vision of several months' duration. On physical examination, the patient was found to have a blood pressure of 230/140 mm Hg. The retinal changes were severe, consisting of extensive deposition of exudate and hemorrhages in the macular area of each fundus, together with tortuosity and beading of the arteries. The urine examination revealed a trace of albumin, occasional erythrocytes and casts. The specific gravity of a concentrated urine specimen (12 hours) was 1.022. Intravenous pyelography revealed no abnormalities. The diodrast and inulin clearances were performed on October 25, 1940. On October 28, the patient was operated upon by Drs. Emile Holman and Frederick Reichert at the Stanford University Hospital. A bilateral resection of the greater splanchnic nerve for approximately 10 cm of its extent with a resection of the 10th to 12th sympathetic ganglia was performed. The recovery was uneventful. The blood pressure remained low for about one week and then rose again to its previous high level. On November 22, 1940, the diodrast and inulin clearances were repeated. At this time the blood pressure was 225/150 mm Hg.

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[1] Peet, M. M., Woods, W. W., and Braden, S., *J. A. M. A.*, 1940, **115**, 1875.

Description of Methods. The diodrast and inulin clearances were performed simultaneously exactly as described in a previous report.[2] The blood pressure determination was made at the beginning, middle and end of each clearance test. The filtration fraction was computed as the $\frac{\text{inulin clearance in cc per minute}}{\text{diodrast clearance in cc per minute}}$. The whole blood flow through the kidney was obtained by the division of the diodrast clearance by the hematocrit determined plasma volume percentage. All determinations were corrected to 1.73 square meters of surface area. In Table I, the average renal clearance (inulin and diodrast) values found in 6 normal men are given.

Results. In the patient, L. G., the renal blood flow was 367 cc per minute before the operation and 420 cc per minute 10 days following the operation. When compared to the average normal renal

TABLE I.
Renal Clearance Determinations in Malignant Hypertension Before and After Splanchnicectomy.

Case No.		Plasma clearance (diodrast) (cc/min.)	Renal blood flow (cc/min.)	Inulin clearance (cc/min.)	Filtration fraction %
	Normal values (avg)*	761	1288	124	17.8
I	Before splanchnicectomy	283	367	108.5	44.5
	After ”	252	420	89.5	35.7
II	Before ”	287	386	88.2	33.9
	After ”	210	304	92.5	44.0

*These figures represent the average clearance values of 6 normal men.

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[2] Friedman, M., Selzer, A., and Rosenblum, H., *J. Clin. Invest.*, 1941, **20**, 2.

blood flow (Table I), it can be seen that there was a great reduction in the blood flowing through the kidneys of this patient both before and after splanchnicectomy. The inulin clearance decreased considerably from 108.5 before to 89.5 cc per minute after operation. It is unknown whether this was due to the splanchnicectomy or to the possible progressive glomerular damage that might have occurred in the 3 months which elapsed between the 2 clearance determinations. The filtration fraction also fell, presumably because of the fall in the inulin clearance, but it will be observed that it was still about twice the normal value.

In the patient, F. P., there was no significant change in the renal blood flow, for it was 386 cc per minute before splanchnicectomy and 304 cc per minute 27 days after the operation. The inulin clearance remained about the same. The filtration fraction rose from 33.9% to 44.0%.

Summary. The renal blood flow (diodrast) and the glomerular filtration rate (inulin) were measured in 2 patients with hypertension before and after bilateral splanchnicectomy. There was no significant change in the renal blood flow in either case. The glomerular filtration rate decreased in one patient and remained the same in the other.

The authors wish to express their thanks to Drs. T. Addis, E. Holman and F. Reichert of Stanford Medical School, and to Dr. H. C. Naffziger of the University of California Medical School, for their aid in making these cases available for study.

13345

Effect of Oral Administration of Iodoacetic Acid on Cystine Content of Rats.*

Abraham White and Elizabeth Simon Stevenson.

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Growth studies in rats have demonstrated[1] that orally administered iodoacetic acid exerts a marked influence on sulfur metabolism, probably by imposing on the organism an increased demand for the sulfur-containing amino acids, cystine and methionine, or for substances behaving like these two amino acids in metabolism. The greater requirement of the rat for the above-mentioned sulfur-containing compounds, when iodoacetic acid is added to the basal diet, has been interpreted as evidence that the halogen-containing acid is detoxified in the organism by a mechanism involving cystine and methionine or closely related metabolically important substances. It seemed evident that if the latter working hypothesis is correct, the administration of iodoacetic acid to rats should deplete the total available sulfur reserves of the body tissue and, more specifically, should diminish the total sulfhydryl content of the animals, inasmuch as this grouping would likely function in reactions involving the detoxication of iodoacetic acid, *e. g.,* mercapturic acid formation.

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* Aided by a grant from the Fluid Research Fund of the Yale University School of Medicine.

[1] Stevenson, E. S., and White, A., *J. Biol. Chem.*, 1940, **134**, 709.

The experiments to be described demonstrate that the total sulfhydryl content of rats ingesting a basal diet plus added iodoacetic acid is significantly less than that of control rats ingesting the basal diet alone or that of animals receiving the iodoacetic acid-containing ration plus supplements of either cystine or methionine.

Procedure. The diets employed and the care of the animals have been described.[1] Young rats at weaning were placed in individual cages and given the basal diet *ad libitum* plus a daily supplement of 400 mg of dried yeast (Northwestern Yeast Co.). Three groups of animals, 20 in each group, were employed. When the animals had reached a body weight of approximately 80 g, one group was sacrificed to furnish data for normal control rats. The other 2 groups were placed on a basal diet containing iodoacetic acid (100 mg of recrystallized iodoacetic acid added to each 100 g of basal diet[1]) for a period of 28 to 31 days. At the end of this time, one of these 2 groups of animals was sacrificed; these rats furnished representative values for the effects of feeding iodoacetic acid. Of the last group, half of the animals were given the iodoacetic acid-containing basal diet, to which was added 450 mg of *dl*-methionine per 100 g of diet. The remaining animals were given the iodoacetic acid-containing basal diet, to each 100 g of which had been added 360 mg of *l*-cystine. Following a period of 4 weeks on the supplemented diets, these animals were sacrificed and furnished representative values for the effects of feeding a sulfur-containing amino acid together with the iodoacetic acid.

The average daily growth rates and food consumptions for the animals employed in this study were very similar to those which have been reported[1] in the growth studies. After the animals had been sacrificed, each was treated as described by Beach and White[2] for total cystine analysis. The body weight was corrected for the weight of the gastrointestinal tract and contents. Pelts and carcasses were hydrolyzed and analyzed separately, as described,[2] with the estimation of total cystine being conducted by the method of Graff, Maculla, and Graff.[3] Final analysis of the cuprous mercaptide precipitate was made by the micro-Kjeldahl method.

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[2] Beach, E. F., and White, A., *J. Biol. Chem.*, 1939, **127**, 87.

Although each carcass and pelt was analyzed separately, the final total cystine values have been calculated per gram of total animal. This was considered advisable since the rats ingesting the iodoacetic acid-containing basal diet alone had very small amounts of subcutaneous lipids. This resulted in a marked relative increase in the weight of the pelts of the animals in the other two groups and made a comparison on the basis of unit of body weight somewhat more difficult. It has also seemed more logical to regard the analytical values as representing total sulfhydryl rather than total cystine, even though the latter amino acid represents the major portion of the total sulfhydryl of the organism, *i. e.*, in the form of cystine, cysteine, and glutathione, either free or in the body proteins. However, the possible occurrence of other sulfhydryl compounds, precipitable under the conditions of analysis, cannot be ignored.

Table I contains the average values for the total sulfhydryl (calculated as cystine) content of each of the groups of animals. The standard deviations were also calculated and are included in the table. It will be seen that the total sulfhydryl content of the animals ingesting the iodoacetic acid-containing basal diet is definitely less than that of the control group on the basal diet alone. Moreover, the superimposition of either a cystine or a methionine supplement on the iodoacetic acid-containing basal diet not only relieves the inhibition in growth induced by the halogen aliphatic acid, but also permits the maintenance of a greater than normal concentration of total sulf-

TABLE I.
Average Values for Total Sulfhydryl Content of Animals Fed Iodoacetic Acid With and Without Cystine and Methionine Supplements.

	Diet of animals*	Total sulfhydryl† per g of body wt, mg
Group A	Basal diet	3.4 ± 0.10
Group B	" " + iodoacetic acid	2.5 ± 0.14
Group C	" " + " " + cystine supplement	4.3 ± 0.26
Group D	" " + " " + methionine supplement	4.7 ± 0.21
Group E	All animals in groups C and D	4.5 ± 0.25

* The averages in groups A and B are each based on 20 animals, and in C and D on 10 animals in each group.

†Calculated as cystine.

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[3] Graff, S., Maculla, E., and Graff, A. M., *J. Biol. Chem.*, 1937, **121**, 81.

hydryl reserves in the body tissues, even in the face of a dietary adjunct (iodoacetic acid) which draws upon these reserves.

In order to determine whether the differences between the several classes of experimental animals were significant, the data have been treated statistically and the P value calculated by the method of Fisher[4] for small series. These values are presented in Table II. It will be seen that there is a significant difference between the sulfhydryl content of normal animals ingesting the basal diet alone and of rats given the same diet to which had been added iodoacetic acid. There is also a significant difference between the analytical values obtained for the animals on the iodoacetic acid-containing basal diet and those found for the rats ingesting this diet supplemented with either cystine or methionine. Finally, it will be observed from the standard deviations and the probability calculations that there is no significant difference between the effects of the supplementary feeding of *l*-cystine and of *dl*-methionine. This latter result would be predicted by the fact that the growth-stimulating effects of either of these sulfur-containing amino acids, added to a growth-inhibitory iodoacetic acid-containing basal diet, are approximately of the same magnitude.[1]

TABLE II.
Significance of Average Values for Sulfhydryl Concentration.

Group averages compared*	P value†
Normal animals vs. iodoacetic acid-fed animals (Group A vs. Group B)	<0.01
Iodoacetic acid-fed animals vs. cystine and methionine supplemented animals (Group B vs. Groups C + D)	<0.01
Normal animals vs. cystine and methionine supplemented animals (Group A vs. Groups C + D)	<0.01
Cystine supplemented animals vs. methionine supplemented animals (Group C vs. Group D)	0.29

*Designation of groups as in Table I.
†P values of 0.05 or less are considered to indicate significant differences.

Summary. The addition of iodoacetic acid to a low-protein basal diet results in a statistically significant lowering of the total sulfhydryl (calculated as cystine) content of rats ingesting this diet, as contrasted to animals given the basal diet alone. The supplementing of the iodoacetic acid-containing basal diet with either *l*-cystine or *dl*-methionine prevents the lowering in total sulfhydryl concentration in the tissues of rats and, indeed, permits a final sulfhydryl content higher than

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[4] Fisher, R. A., *Statistical Methods for Research Workers*, 1932, 4th Ed., Oliver and Boyd, London.

that found for control animals fed the basal diet alone. These data would appear to support the conclusion that orally administered iodoacetic acid exerts an influence on the sulfur metabolism of the organism.

13346

Effect of Chlorin-e-Rhodin-g on Experimental Tuberculosis.*

HOWARD J. HENDERSON AND ESMOND R. LONG.
From the Henry Phipps Institute, University of Pennsylvania.

Inhibition of growth of human type tubercle bacilli (H 37) and avian tubercle bacilli in cultures on glycerol broth and Sauton's medium by certain chlorophyll compounds was reported by Daly, Heller and Schneider.[1] The compounds used included chlorin-e and chlorophyllin-a, the relationship of which to chlorophyll is described in the article cited. Unpublished experiments by the same authors indicated that rhodin-g, a compound related to chlorophyll-b in the same way that chlorin-e is related to chlorophyll-a, has bacteriostatic properties for tubercle bacilli similar to those of chlorin-e.

Chlorin-e and rhodin-g occur in combination in the proportion of approximately 2.75 to 1 in the breakdown of chlorophyll, preserving the same relationship that chlorophyll-a and chlorophyll-b have in the green plant. Since they have similar bacteriostatic effect, separation seemed unnecessary for further experiments on their inhibitory effect on tubercle bacilli.

Our own experiments have confirmed those previously reported. Human type (strain DT) and bovine type (strain 523) tubercle bacilli were inhibited in culture in glycerol broth and Long's medium by the sodium salt of chlorin-e-rhodin-g in concentrations from 0.025 to 0.01%, the effect of the higher concentration being greater than that of the lower.

An attempt was next made to determine if the sodium salt of chlorin-e-rhodin-g inhibits the development of tuberculosis *in vivo*.

Preliminary trial showed that guinea pigs were not suitable. Parenteral administration was desirable, and experiment showed

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* This work was supported by a grant from the Jovan Laboratories, Inc., New York, N.Y.

[1] Daly, S., Heller, G., and Schneider, E., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 74.

that the pigment was poorly absorbed when given intraperitoneally or subcutaneously. Accordingly the method chosen was intravenous injection in rabbits.

Method. Twenty-three young male rabbits were infected with 0.01 or 0.001 mg Ravenel strain bovine type tubercle bacilli and given daily injection of 55 mg pigment dissolved in water. The treatment was well tolerated. Twenty rabbits were infected with the same dose of bacilli but not treated with the pigment. Seven rabbits received the pigment but were not infected. The total amount of pigment injected in the tuberculous rabbits ranged from 2.4 g in the animals killed at 72 days to 6.7 g in a rabbit killed at 169 days. A few animals died of intercurrent disease in the course of the experiment, but the majority were killed at intervals. The tuberculous rabbits, both pigment-treated and nontreated, were killed in 3 main groups, viz., at about 2½ months, 3½ months and 5 months.

Necropsy was performed on all animals, and the gross condition of the organs noted. Microscopic sections, stained with hematoxylin and eosin and by the Ziehl-Neelsen technic for tubercle-bacilli, were cut routinely of the lungs, liver, spleen, one kidney and bone marrow of the femur. Serial blood counts were made on a number of rabbits of each series.

Results. In a few pairs of rabbits dying or killed early in the course of the experiment the amount of tuberculosis appeared to be less in the pigment-treated than in the control infected, untreated rabbits. However, the difference was slight and after a brief initial period no significant differences were noted between the infected pigment-treated and infected control animals. In practically all animals of each series the lungs, spleen and kidneys were grossly tuberculous, the extent of involvement increasing with the duration of the disease. No constant differences in the state of these 3 organs in the 2 series were seen. Microscopically the bone marrow was found tuberculous in a large percentage of cases in each group. In the sections examined tubercles were found in 12 of 22 examined bone marrows in the pigment-treated animals, and in 6 of 19 examined cases in the nontreated animals. The difference in the two groups is probably not significant. The sections were small, and where tuberculosis was found in the bone marrow it was not more severe in the pigment-treated than the control animals.

No differences were observed in the 2 series in weight. In each group, as well as in the pigment-treated noninfected group, an initial increase in weight, normal for the age of the animals, occurred. This lasted 2 months. During the next month a drop occurred. A sharp secondary increase in weight occurred in each group during the fourth month. After this a rapid loss in weight occurred.

The chief change, other than the tuberculous involvement, was the heavy pigmentation in the pigment-treated animals. The liver and spleen were grossly much darker than in the untreated rabbits, and the bone marrow had a deep chocolate color. Microscopic sections showed large numbers of phagocytic cells filled with golden-brown granules in the bone-marrow and spleen. The pigment was readily differentiated from blood pigment by the Prussian blue reaction. In both the spleen and bone marrow the pigment stood out more conspicuously in Ziehl-Neelsen than in hematoxylin and eosin stains. In the liver the pigment was frequently found in the Kuppfer cells, but was much less granular than in the spleen and bone marrow, the cells often having a homogeneous brown color. Pigment was frequently visible as a dark brown mass in the bile canaliculi.

Pigment was commonly observed in phagocytic cells around tubercles. However, the pigmented cells did not take part in the palisade of epithelioid cells forming the true border of tubercles. Pigment and acid-fast bacilli were occasionally found in the same phagocytic cell, but this was the exception. Whether the 2 cells were fundamentally of the same origin or not, a distinct difference in functional activity was evident, large numbers of cells containing pigment and other large numbers containing bacilli.

Summary. Chlorin-e-rhodin-g inhibited the growth of tubercle bacilli *in vitro,* but had no effect on the course of experimental tuberculosis in rabbits. The pigment accumulated in large quantity in phagocytic cells in the spleen and bone marrow. Pigmented cells were frequently concentrated on the border of tubercles, but did not appear to function as epithelioid cells and rarely were found containing tubercle bacilli.

13347

Accumulation of Chlorophyll Pigments in Visceral Organs and their Elimination.*

HOWARD J. HENDERSON AND ESMOND R. LONG.
From the Henry Phipps Institute, University of Pennsylvania.

Reference has been made[1] to the accumulation of pigment in the spleen, bone marrow and liver after intravenous injection of chlorin-e-rhodin-g in rabbits. It seemed desirable to determine if the accumulated pigment is eliminated in the course of time and if other chlorophyll pigments are similarly deposited.

Five normal adult male rabbits were injected intravenously with sodium chlorin-e-rhodin-g for 34 days in daily doses of 145 mg in 14.5% solution. At the end of this period, when each rabbit had received 4.44 g of the pigment, 2 rabbits were killed. The other 3 were allowed to live 3 months longer, during which no pigment was injected. They were then killed and the amount of pigment in the tissues was compared with that in the other 2 animals.

In the 2 rabbits killed after 34 days of pigment injection the spleen and liver were very dark and the bone marrow was deep chocolate in color as in previous experiments. Microscopically golden-brown pigment was conspicuous in large phagocytic cells in the spleen and bone marrow, and was found in smaller amount in the liver as previously described. In the 3 rabbits treated identically with the pigment, but allowed to live 3 months with no pigment injection, the organs, including the bone marrow, were grossly much lighter when seen at necropsy. This suggested that the pigment had been eliminated to a considerable extent. However, microscopic examination showed that large amounts still persisted in the phagocytic cells of the 3 organs named. It was estimated from various fields in histological specimens that half to three-quarters of that present at the end of the month of treatment persisted in the spleen and bone marrow after 3 months without treatment. In the spleen the pigment was loosely arranged in very large phagocytic cells and at times appeared to lie free in tissue spaces. In the bone marrow the phagocytic cells containing pigment were sharply

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* This work was supported by a grant from the Jovan Laboratories, Inc., New York, N.Y.

[1] Henderson, H. J., and Long, E. R., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 435.

defined and the pigment was present in the form of coarse dense granules.

The amount in the liver appeared to have decreased to a greater extent than in the spleen and bone marrow. Few Kuppfer cells containing pigment were found, but many large phagocytic cells containing pigment were seen in the perivascular tissue, especially in the portal triads, a fact suggesting migration of pigmented cells in the lymph. Whether any of the pigment presumably originally present in the Kuppfer cells had been eliminated in the bile could not be told by histological methods.

The pigment was not found in the kidneys, lungs, myocardium, adrenals, alimentary tract, generative organs or central nervous system, but was present in considerable amount in lymph nodes.

Blood counts on animals injected with large amounts of sodium salt of chlorin-e-rhodin-g did not show any alteration from the normal blood picture in serial examination during the course of administration of the pigment, although much pigment was found in the bone marrow of these animals at necropsy.

Summary. Chlorin-e-rhodin-g was very slowly eliminated from the tissue of rabbits after intravenous injection. The major portion of that deposited in the spleen and bone marrow after a month of administration was still present in phagocytic cells 3 months later. Somewhat greater elimination occurred from the liver. The deposit in the hemopoietic organs had no evident effect on their function.

13348

Effect of Stilbestrol on Lactogenic Content of Pituitary and Mammary Glands of Female Rats.*†

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Estrogenic treatment of male and female rats has been shown to cause an increase in the lactogenic content of the pituitary (Reece and Turner[1]). This increase amounted to 45% after treatment with

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* Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 781.

† This study has been aided in part by a grant of the International Cancer Research Foundation.

[1] Reece, R. P., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bul.* 266, 1937, 64.

estrone and as much as 229% after estradiol benzoate administration. Milk secretion was initiated, at least in some cases, from lobule-alveolar glands.

Reece and Mixner[2] found that 200 γ of testosterone propionate injected per day for 15 days into mature spayed rats caused an increase of 41% in lactogen content per pituitary. Pituitary weight was not changed. The treatment induced an extensive development of the lobule-alveolar system of the mammary glands and initiated secretion.

Androsterone, which does not cause mammary growth,[3] was found to cause no significant change either in pituitary weight or pituitary lactogen content in mature spayed female rats.[4] Desoxycorticosterone, which does cause mammary hyperplasia,[5] nevertheless caused no increase in pituitary lactogen content in male and female guinea pigs in spite of a significant increase in size.[6]

Stilbestrol has been shown to be at least as active as estradiol benzoate and more active than estrone on the hyperplasia of the mammary glands of mice[7] and genital organs of rats.[8] It was of interest to compare its effects with that of the natural estrogens on the lactogen content of the pituitary and on the mammary glands of mature spayed rats. It was also thought that a substantial increase in pituitary lactogen content on stilbestrol[‡] administration might help to explain the copious lactation which has been shown to occur from the use of this drug in the virgin and dry goat.

Methods. Mature, multiparous rats were spayed and allowed to recover from the operation for 2 weeks. They were then given 10 daily subcutaneous injections of stilbestrol (4:4 dihydroxy $\alpha\beta$ diethyl stilbene) dissolved in oil. On the eleventh day they were sacrificed and the pituitaries removed and weighed. The mammary glands were fixed in Bouin's fluid and prepared as whole mounts. Representative glands were sectioned. The lactogenic content of the pituitaries was assayed by the minimum-micro technic and calculated to international units on the basis of the work of Meites, *et al.*[9]

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[2] Reece, R. P., and Mixner, J. P., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 66.

[3] Nelson, W. O., and Merkel, C. G., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 823.

[4] Reece, R. P., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 265.

[5] Van Heuverswyn, J., Folley, S. T., and Gardner, W. U., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 389.

[6] Turner, C. W., and Meites, J., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 234.

[7] Lewis, A. A., and Turner, C. W., *Cancer Research*, 1941, **1**, 55.

[8] Sondern, C. W., and Sealey, J. L., *Endocrinology*, 1940, **27**, 670.

[‡] Stilbestrol was kindly supplied by Dr. D. F. Green of Merck & Co., Rahway, N. J.

Results. The weight of the pituitaries from the multiparous control rats was considerably higher than that found by Reece and Turner[1] in mature virgin(?) rats (14 mg and 7.5). Lactation has been shown to cause a permanent increase in pituitary size in rabbits[10] and cattle.[11]

A dosage of 3.2 γ per day of stilbestrol caused a 21% increase in pituitary size and 29% increase of lactogen per 100 g body weight over that found in the untreated females (Table I). The content per mg of pituitary was not changed. A dosage of 10 γ per day was still more effective causing an increase of 44% in pituitary weight and 155% in i.u. of lactogen per pituitary. The content per mg of pituitary was increased 111% and per 100 g body weight, 184%. A dosage of 20γ per day was still more effective in increasing pituitary size (105%), content per pituitary (210%), and per 100 g body weight (226%) but gave a lower increase in content per mg of pituitary (79%). The pituitary lactogen in the groups of rats given 100 γ and 200 γ of stilbesterol was considerably higher than that found by Reece and Turner,[1] and Reece, *et al.*,[12] in the pituitaries of normal lactating rats soon after parturition.

TABLE I.
Lactogenic Content of Pituitaries of Stilbestrol Treated Multiparous Spayed Rats.

				Pit. wt		I.U. of lactogen					
No. of animals	Wt autopsy, avg, g	Days treated	Daily dosage, γ	mg	increase, %	per pit.	increase, %	per 100 g body wt	increase, %	per mg pit.	increase, %
12	238	0	0.0	14.0		.29		.122		.028	
12	223	10	3.2	16.9	21	.35	21	.157	21	.028	0
6	214	10	10.0	20.1	44	.74	155	.346	184	.059	111
5	226	10	20.0	28.7	105	.90	210	.398	226	.050	79

According to these results, stilbestrol appears to be more effective than estrone per gamma in increasing the lactogen content of the pituitary in the rat for Reece and Turner[1] secured a 45% increase per pituitary with a dosage of 50 γ per day of estrone. With 100 γ daily of estradiol benzoate they found a 73% increase in lactogen per pituitary after 18 days' treatment. After 6 to 14 days' treatment with 20 γ daily of estradiol benzoate the increase per pituitary was 229% which is slightly higher than we secured with stilbestrol

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[9] Meites, J., Bergman, A. J., and Turner, C. W., *Endocrinology*, 1941, **28**, 707.

[10] Bergman, A. J., and Turner, C. W., *Endocrinology*, in press.

[11] Lewis, A. A., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bul.* 310, 1939.

[12] Reece, R. P., Hathaway, I. L., and Davis, H. P., *J. Dairy Sci.*, 1939, **22**, 1.

(210%). However, Meites and Turner[13] secured a 410% increase in pituitary lactogen content of guinea pigs given a total of 3000 i.u. of estrone in 15 days. They also secured a comparable increase on stilbestrol administration.

The mammary glands from the control group of rats consisted, in half the cases, of fully developed duct systems. Several of these had only main and secondary ducts while others had interlobular ducts well developed. The other control rats had from slight lobule development to, in two cases, fairly well developed alveolar lobules. In the latter cases the alveoli were unhypertrophied.

In the group of rats given 32 γ of stilbestrol there was also considerable variation in the development of the mammary glands but the range was from early to heavy lobule development. In 6 cases alveolar lobules were extensively developed. These alveoli were hypertrophied, indicating fair secretion.

The group of rats given 100 γ of stilbestrol did not have any greater development of lobules but the alveoli in all cases were hypertrophied with secretion. The mammary glands from rats given 200 γ of stilbestrol were more variable in regard to alveolar hypertrophy, resembling the group given 32 γ.

Reece and Turner[1] reported the secretion of milk in mammary glands from spayed female rats treated with estradiol benzoate. In this study all of the rats given 100 γ of stilbestrol were secreting milk at autopsy. In these cases there were many alveoli well hypertrophied and filled with milk. In other cases serous secretion had been initiated.

Discussion. The administration of stilbestrol by injection[14] or percutaneous application to the udder[15] has been shown to readily cause copious lactation in virgin and dry goats. This treatment was also shown to cause lobule-alveolar development in virgin goats.[16] In this study 10 days' treatment with stilbestrol was shown to cause milk secretion in some mature spayed female rats. This lactation was of slight extent compared with that found after parturition, however, perhaps because of the short period of treatment and incomplete development of the mammary glands. As an explanation of this milk secretion, it was found that the lactogenic content of the pituitaries of these rats was increased as much as 226% over that found in non-lactating untreated rats.

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[13] Meites, J., and Turner, C. W., unpublished.

[14] Lewis, A. A., and Turner, C. W., *Proc. Am. Soc. An. Prod.*, 1940, p. 63.

[15] Folley, S. J., Watson, H. M. S., and Bottomley, A. C., *J. Physiol.*, 1940, **98**, 1.

[16] Lewis, A. A., and Turner, C. W., *J. Dairy Sci.*, 1941, **24**, 845.

Summary. Stilbestrol treatment of mature spayed rats for 10 days caused serous or milk secretion from partially developed lobule-alveolar glands. The lactogenic hormone content of the pituitaries per 100 g body weight was increased as much as 226% through an increase in size of the pituitary and content per milligram.

13349

Influence of Environmental Temperature on Growth of Mammary Lobule-Alveolar System.*†

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Many studies have been reported indicating that variations in such environmental factors as light, temperature, and diet influence the secretion of the anterior pituitary hormones. In several instances it has been shown these same factors do not adversely influence the ability of the end organs such as the gonads and the thyroid to respond to their growth stimuli. Thus in the case of underfeeding, the testes[1] and the thyroid[2] are responsive to less gonadotropic and thyrotropic hormone respectively than the glands of animals on a normal ration.

Recently, Trentin and Turner[3] showed that the amount of estrogen required to stimulate the growth of the mammary duct system increased as the food intake was decreased. In the light of previous work it was suggested that in this case also the inanition might decrease the responsiveness of the pituitary to estrogen stimulation resulting in the production of a much smaller amount of the pituitary mammogenic duct growth factor. The responsiveness of the pituitary would thus be involved rather than the responsiveness of the end organ, the mammary gland.

During periods of high environmental temperature in the summer of 1941, it was observed that progesterone alone and with estrogens failed to stimulate the same degree of mammary lobule-alveolar de-

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* Aided in part by a grant from the International Cancer Research Foundation.

† Contribution from the Department of Dairy Husbandry, Missouri Agricultural Experiment Station, Journal Series No. 782.

[1] Breneman, W. R., *Endocrinology*, 1940, **25**, 1094.

[2] Stevens, D. J., *Endocrinology*, 1940, **26**, 490.

[3] Trentin, J. J., and Turner, C. W., *Endocrinology*, in press.

velopment as that obtained earlier in the spring. It thus appeared that high environmental temperature as well as inanition might influence mammogen secretion by the anterior pituitary.

It seemed of interest, therefore, to compare the influence of 2 environmental temperature levels upon the responsiveness of the mammary lobule-alveolar system to progesterone[‡] (plus estrogen) and an anterior pituitary extract which contained the mammogenic lobule-alveolar growth factor. According to the pituitary-mammogen theory of mammary gland growth[4, 5] progestin (with estrogen) acts through the pituitary to cause an increased secretion there of the mammogenic lobule-alveolar growth factor, which directly stimulates mammary lobule-alveolar growth.

Procedure. Both progesterone and the pituitary initial extract, each with estrone, were assayed for their mammary lobule-alveolar proliferating properties by a technic recently described.[6] The percentage of ovariectomized mice showing a minimum amount of lobule-alveolar growth after 10 daily subcutaneous injections was used as the criterion of response. Olive oil was used as the carrier for the progesterone and estrone. The pituitary initial extract was prepared according to a method described by Bergman and Turner.[7] A uniform grain ration was fed to all mice *ad libitum.* The assay mice were maintained in constant temperature chambers regulated to 25° and 35°C respectively.

Results. At the 35°C temperature approximately 50% of the

TABLE I.

Lobule-Alveolar Responses of Castrate Female Mice Kept at Different Environmental Temperatures to Injections of Progesterone and a Pituitary Extract.

No. of mice	Injection materials	Total dosage	Temperature °C	Results +	Results —	% Positive
29	Progesterone Estrone	1.25 mg 75 I.U.	25	15	14	51.7
15	Progesterone Estrone	1.25 mg 75 I.U.	35	1	14	6.7
15	Pituitary extract Estrone	12.5 mg 75 I.U.	25	13	2	86.7
8	Pituitary extract Estrone	12.5 mg 75 I.U.	35	7	1	87.5
14	Pituitary extract Estrone	17.5 mg 75 I.U.	25	13	1	92.9
8	Pituitary extract Estrone	17.5 mg 75 I.U.	35	8	0	100.0

[‡] Progesterone was kindly supplied by Dr. E. Schwenk, Schering Corporation.

[4] Gomez, E. T., and Turner, C. W., *Missouri Agr. Exp. Sta. Res. Bul.* 259, 1937.

[5] Mixner, J. P., Lewis, A. A., and Turner, C. W., *Endocrinology,* 1940, **27**, 888.

[6] Mixner, J. P., and Turner, C. W., *Endocrinology,* 1941, **29**, 324.

[7] Bergman, A. J., and Turner, C. W., *J. Biol. Chem.,* 1938, **123**, 474.

animals on both progesterone and the pituitary initial extract died during the assay period. Only surviving animals are included in the data presented in Table I.

At the more normal temperature of 25°C, 1.25 mg of progesterone and 75 i.u. of estrone stimulated lobule-alveolar growth in 51.7% of the animals. This is considered a unit response. On the same level of hormone injection but at 35°C the response dropped to 6.7%, confirming our earlier observations at a high but variable environmental temperature. Thus the high temperature adversely affected the ability of the anterior pituitary to secrete the mammogenic lobule-alveolar growth factor in response to the stimulation of progesterone and estrone. This is in contrast to the results secured with the pituitary initial extract. In this case at the 12.5 mg and the 17.5 mg dosage levels, the percent of positive responses were about equal in both the 25° and 35°C groups, the higher temperature in no way inhibiting the ability of the mammary glands, as end organs, to respond to their direct growth stimulus. The results secured and the suggested explanations fit very well with the pituitary-mammogen theory of mammary gland growth, and these experiments are interpreted as adding further indirect evidence for the validity of this theory.

Summary. Ovariectomized virgin female mice kept at a high environmental temperature (35°C) had a decreased ability to respond to progesterone and estrone injections with mammary lobule-alveolar growth. This same high temperature did not inhibit this response in animals injected with a pituitary extract containing the mammogenic lobule-alveolar growth factor which directly stimulates mammary gland growth. Thus the anterior pituitaries of animals kept at a high environmental temperature have a decreased ability to respond to the stimulus of progesterone plus estrone with the secretion of the mammogenic lobule-alveolar growth factor.

13350 P

Survival of Infant and Adult Rats at High Altitudes.*

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Previous work has disclosed that the young of many species are more resistant to anoxia than are the adult animals.[1] In that investigation the oxygen of the inspired air was displaced by other gases. The present communication reports a comparative study of the effects of high altitude on the survival of adult and infant rats.

Method. The rats were placed in a large decompression chamber and the air evacuated until a pressure equivalent to that at 40,000 feet was attained, the rate of ascent being 2,000 feet per minute. The animals were observed through a window.

Results. From the table it may be seen that after an altitude of 40,000 feet is reached the adult rats succumb rapidly to the low oxygen tension, the survival period being from approximately 10 to 30 minutes. Two rats, 1½ days of age, were removed alive after 150 minutes of exposure at 40,000 feet. The 3-day-old rats survived from less thn 70 minutes to periods longer than 114 minutes. As the animal increases in age its tolerance diminishes as is indicated by the survival periods for 6-day-old rats, the survival period of these rats varying from approximately 16 to 46 minutes.

TABLE I.
Altitude and Survival.

Adult rats			Infant rats			
No. obs.	Survival in min.	Remarks	Age days	No. obs.	Survival in min.	Remarks
3	9	Succumbed	1½	2	150	Alive
2	10	’’	1½	1	135	Succumbed
1	13	’’	3	3	107	Alive
3	18	’’	3	4	114	Succumbed
3	30	’’	3	1	91	’’
			3	1	80	’’
			3	1	71	’’
			6	4	46	’’
			6	2	16	’’

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* Aided by a grant from the Child Neurology Research (Friedsam Foundation). We are indebted to Dr. Alvan L. Barach for the use of the decompression chamber.

[1] Fazekas, J. F., Alexander, F. A. D., and Himwich, H. E., *Am. J. Physiol.*, 1941, **134**, 281.

Summary. Infant rats can tolerate a low oxygen concentration (3.87%) at high altitudes (40,000 feet) longer than adults of the same species. This tolerance decreases with age and is probably associated with the lower cerebral metabolism of infant brain.[2] The diminution of metabolic rate of adult rats by thyroidectomy increases the resistance of these animals to high altitudes.[3] The importance of the factor of aeroembolism in this differential survival has not been determined.

13351 P

Recovery of Pupillary Function in Cut Optic Nerves of Cats and Monkeys.*

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The question of structural and functional recovery of central nervous system tissue in mammals is still a moot point. There are many experiments dealing with regeneration of the optic nerve in fish but practically none in higher forms. Sugar and Gerard[1] studied regeneration of the spinal cord in the rat and found that "true anatomical and physiological regeneration can occur in the rat spinal cord."

We studied the recovery of the pupillary light reflex after sectioning the optic nerves in 3 cats and 4 monkeys by the pupillographic method as developed by Lowenstein.[2] The recordings were made in the dark with infra-red sensitive film. The film was then projected on a screen and pupillary diameter measurements made in tenths of mm. The time intervals were in tenths and seconds.

From 16 to 17 months after the (right) optic and ciliary nerves were severed just behind the globe, direct (of the operated eye) and

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[2] Himwich, H. E., Sykowski, P., and Fazekas, J. F., *Am. J. Physiol.*, 1941, **132**, 293.

[3] Barach, A. L., Eckman, M., and Molomut, N., *Am. J. Med. Sci.*, 1941, **202**, 336.

* This work has been aided by grants from the Josiah Macy, Jr., Foundation and the Rockefeller Foundation for Medical Research.

[1] Sugar, O., and Gerard, R. W., *J. Neurophysiol.*, 1940, **3**, 1.

[2] Lowenstein, Otto, *Arch. Psychiat. Nervenkr.*, 1927, **82**, 285.

consensual (of the normal eye) constriction was found when a light of 60 watts at a distance of 1 meter was projected on the experimental (right) eye. Great care was taken to insure that no amount of light would reach the normal (left) eye. In several experiments a heavy bandage was placed on the normal eye; in the other cases where the consensual reaction of the other eye was to be recorded, this eye was carefully protected against direct stimulation. The direct pupillary response was found to be minimal-achange from 0.1 to 0.5 mm. The consensual response, *i. e.,* from the experimental eye to the normal eye was slightly more conspicuous, showing constriction of 0.5 to 1.5 mm, while the consensual reaction from the normal to the operated eye manifested by a decrease of 2.0 to 3.0 mm in the pupillary diameter.

Our experiments indicate that there is some restitution of function of afferent fibres going to the pupillary centers in the cut optic nerve which actually is central nervous tissue. Anatomic studies have not been completed.

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Bone Calcium During Hyperthyroidism.

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Parhon[1] observed an increased excretion of calcium in rabbits fed thyroid gland. This increased excretion of calcium has also been reported by other authors.[2-5] Aub, *et al.,*[6] have also observed an increased excretion of calcium in the feces of hyperthyroid patients. Others have demonstrated, by the use of roentgenograms, a decalcification in patients with hyperthyroidism. In order to account for the increased calcium excretion in thyroid-fed animals and for the osteoporosis observed in human hyperthyroidism, Aub, *et al.*,[6, 7] assumed that an excess of thyroid has a specific catabolic action on the calcium deposits in bone, leading to an increased mobilization and excretion of calcium. However, Hansman and Wilson[8] and Beaumont, *et al.*,[9] have shown that the calcium and phosphorus balance may be positive during thyrotoxicosis. In 6 cases (Beaumont, *et al.*) the calcium excretion remained the same after as before successful operation. These authors believe that some other cause than a specific catabolic effect of an excess of thyroid on bone must be sought to explain the increased calcium excretion and decalcification that is seen in some patients with Grave's disease. Hansman and Wilson, in a review of the literature, and from their own studies, favor the view that an associated hyperparathyroidism is the direct cause of an increased mobilization and excretion of calcium.

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[1] Parhon, M., *Mem. Soc. de biol.,* 1912, **72**, 620.

[2] Kummer, R. H., *Rev. Med. de la Suisse Romande,* 1917, **37**, 439.

[3] Hunter, D., *Lancet,* 1930, **1**, 947.

[4] Pugsley, L. I., and Anderson, E., *Biochem. J.,* 1934, **28**, 754.

[5] Cope, O., and Donaldson, G. A., *J. Clin. Invest.,* 1937, **16**, 329.

[6] Aub, J. C., Bauer, W., Heath, C., and Ropes, M., *J. Clin. Invest.,* 1929, **7**, 97.

Little experimental work has been done to lend support to either view. Hoskins[10] reported an increase in the weight of the bones, in both the wet and dry state, of thyroid-fed rats. Smith and McLean[11] studied the ash content of the femur of thyroid-fed rats and concluded that, with a diet adequate in calcium, severe hyperthyroidism does not produce decalcification. Only one report of the calcium content of bones during experimental hyperthyroidism has appeared. Parhon, *et al.*,[12] reported a slight decrease in the calcium content of the bones of thyroid-fed dogs. Their analyses show considerable variation and make their conclusions of doubtful significance. We, therefore, undertook a study of the calcium content of the femur of thyroid-fed rats.

Adult male rats, averaging 250 g in body weight, were fed, *ad libitum,* a diet consisting of: salts (Osborne and Mendel), 4; cod liver oil, 4; Crisco, 10; casein, 20; and cornstarch, 62 parts. A supplement of 200 mg of dried yeast was fed each day. This amount of yeast will not prevent a loss of weight in the thyroid-fed rats.[13, 14] The thyroid-fed rats received 100 mg of desiccated thyroid gland

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[7] Albright, F. W., Bauer, W., and Aub, J. C., *J. Clin. Invest.*, 1931, **10**, 187.

[8] Hansman, F. W., and Wilson, F. H., *Med. J. Australia,* 1934, **1**, 37, 81.

[9] Beaumont, G. E., Dodds, E. C., and Robertson, J. D., *J. Endocrinology,* 1940, **2**, 237.

[10] Hoskins, E. R., *J. Exp. Zool.*, 1916, **21**, 295.

[11] Smith, E. E., and McLean, F. C., *Endocrinol.*, 1938, **23**, 546.

[12] Parhon, C. I., Derevici, H., and Derevici, M., *Compt. rend. Soc. de Biol.*, 1932, **109**, 1396.

[13] Drill, V. A., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 313.

[14] Drill, V. A., and Sherwood, C. R., *Am. J. Physiol.*, 1938, **124**, 683.

per day.* Calcium was determined in the ash of the fat-free femur by the method of Washburn and Shear.[15]

After 25 days of thyroid feeding the rats had lost an average of 56 g, whereas the control rats had gained an average of 12 g in body weight. The rats were then dissected. The percentage of ash in the fat-free femur of the thyroid-fed animals (10 rats) showed a slight, but statistically insignificant drop from the value of the control animals (8 rats). The femur calcium of the thyroid-fed rats varied from 35.28 to 40.19% with a mean value of 38.55 ± 0.616, whereas the control rats varied from 38.08 to 42.31% calcium with a mean of 41.21 ± 0.570. Although the difference of 2.66% between the means is statistically significant, this difference seems slight when it is remembered that the experimental group had lost 23% of their body weight during thyroid feeding.

The diet used was relatively rich in calcium, containing 0.48% calcium. During the experiment the thyroid-fed animals showed an increased food consumption with a consequent increase in calcium intake, which would aid in maintaining a positive calcium balance in the thyroid-fed rats. The high calcium diet and the increase in food intake may have prevented a greater reduction in bone calcium than that actually found. But, at least thyroid feeding does not seem to have had any marked catabolic effect on the bones with a diet adequate in calcium. Other rats on the same diet, which have been fed 100 mg of thyroid gland for 80 days, plus a supplement of vitamin B_1 and yeast to prevent a loss of weight,[14] did not show any detectable osteoporosis upon X-ray examination.

These results, based on the measurement of bone calcium, point to the same conclusions reached by Hansman and Wilson[8] and Beaumont, *et al.*,[9] in clinical studies. The experiments also confirm the conclusion of Smith and McLean,[11] who, in a study of the ash content of the bones of hyperthyroid rats, concluded that with a diet adequate in calcium, severe hyperthyroidism does not produce osteoporosis.

Summary. Rats fed 100 mg of thyroid gland per day showed, after 25 days, an average decrease of 2.66% in the calcium content of the ash of the fat-free femur. The concomitant loss in body weight of 23%, makes it unlikely that the thyroid feeding *per se* had a specific effect on bone calcium.

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* The author is indebted to Dr. C. N. Frey of the Fleischmann Laboratories for the dried yeast and to Dr. Rhodehamel of Eli Lilly and Company for the desiccated thyroid gland.

[15] Washburn, M. L., and Shear, M. J., *J. Biol. Chem.*, 1932, **99**, 21.

13353

Neuroinvasiveness of Poliomyelitis Virus in Relation to Vitamin D Nutrition.*

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Recently, the question has been raised as to whether or not poliomyelitis virus can spread along the peripheral nerves of normal rhesus monkeys. It has been stated that poliomyelitis virus has but a slight affinity for the exposed axons of peripheral medullated nerves and that the myelin surrounding the axis cylinders of healthy monkeys may be regarded as capable of stopping the spread of virus into the central nervous system.[1] It has, furthermore, been claimed that for such spread to occur (*i. e.,* for successful infection) it was necessary that the monkeys be deficient in vitamin D.[1] Thus, it appeared possible that the success with which some investigators (Fairbrother and Hurst,[2] Demme,[3] Hurst,[4] Sabin and Olitsky,[5] Bodian and Howe[6]) obtained infection by the intrasciatic route, as compared with the high incidence of failure reported by others (Flexner and Amoss,[7] Harrison and Woolpert,[8] Toomey,[1, 9]), might depend upon the status of vitamin D nutrition in the inoculated animals. Since we intended to use the intrasciatic route of infection in another investigation, we were led to inquire whether successful infection by this route was dependent on the existence of vitamin D deficiency in the inoculated monkeys.

Experimental. One of us (A.B.S.) has previously observed that monkeys arriving in the laboratory a short time after their long sea voyage to this country may exhibit roentgenologic and histologic evidence of deficiency of vitamins C and D. Guest and Rapoport[10]

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Toomey, J. A., *Am. J. Dis. Child.,* 1940, **60**, 548.

[2] Fairbrother, R. W., and Hurst, E. W., *J. Path. Bact.,* 1930, **33**, 17.

[3] Demme, H., *Deutsch. Z. f. Nervenh.,* 1930, **116**, 156.

[4] Hurst, E. W., *J. Path. Bact.,* 1930, **33**, 1133.

[5] Sabin, A. B., and Olitsky, P. K., *J. Am. Med. Assn.,* 1937, **108**, 21.

[6] Bodian, D., and Howe, H. A., *Brain,* 1940, **63**, 135; PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 170.

[7] Flexner, S., and Amoss, H. L., *J. Exp. Med.,* 1917, **25**, 525.

[8] Harrison, J. A., and Woolpert, O. C., *J. Infect. Dis.,* 1939, **65**, 214.

[9] Toomey, J. A., personal communication.

[10] Guest, G. M., and Rapoport, S., *Physiol. Rev.,* 1941, **21**, 410.

have recently reported that monkeys which had been kept indoors for a number of months on a diet obviously deficient in vitamin D exhibited chemical and roentgen evidence of rickets and osteoporosis. Therefore, in the first tests we used 4 rhesus monkeys which had been out of doors at the dealer's farm for a number of weeks (April, 1940) and then several weeks in our own animal quarters, where they received a diet of whole milk, oranges, bananas, bread and peanuts. Roentgenograms were made of the long bones and the serum calcium and inorganic and organic, acid-soluble phosphorus in the blood were determined to indicate the status of vitamin D nutrition. The significance of the concentrations of organic acid-soluble P in the blood cells of monkeys, in relation to their intake of vitamin D and seasonal exposure to sunlight, has been discussed in a recent review.[10] It has been shown that in these and other animals the concentration of organic acid-soluble P in the blood cells constitutes an index of the state of the labile phosphorus reserves of the body and of phosphorus metabolism as it is influenced by vitamin D.[10, 11] The chemical data on monkeys 3, 5, 7, and 8 listed in Table I are representative of the range of values determined in the bloods

TABLE I.
Development of Poliomyelitis Following Intrasciatic Injection of Virus in Relation to Vitamin D Nutrition.

	Ca and P in blood before inoculation				
Rhesus monkey No.	Ca mg/100 cc	Inorganic P mg/100 cc whole blood	Organic P mg/100 cc cells	Roentgenograms of long bones	Result of intrasciatic inoculation
3	11.0	5.1	54.6	Normal	F_3 Par. 4 Prostr. 6†
5	12.6	4.4	49.5	”	F_3 ” 4 Dead 5
7	11.0	6.2	55.5	”	F_3 ” 3 ” 5
8	11.8	4.9	61.4	”	F_3 ” 3 Prostr. 4
3-39	—	(2.2) 7.8*	(37.6) 85.3*	”	F_3 ” 3 ” 5
3-41	—	(4.4) 6.4*	(46.8) 61.9*	”	F_4 ” 4 ” 5
3-40	—	3.1	47.6	Marked rickets and scurvy	F_5 ” 5 ” 7
3-42	—	2.9	50.9	Slight rickets and scurvy	F_4 ” 4 ” 6

* The initial P values are in parenthesis, while those marked with the asterisk were determined on the 12th day of continuous daily administration of 5,000 U.S.P. units of vitamin D or 2 days before inoculation of virus.

†F_3 Par. 4, Prostr. 6—fever 3rd day, paralysis 4th day, and prostrate 6th day after inoculation.

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[11] Rapoport, S., and Guest, G. M., *J. Biol. Chem.*, 1938, **126**, 749.

of normal monkeys and the roentgenograms of their long bones were normal. Each of these monkeys received an inoculation of 1 cc of a 10%, lightly centrifuged suspension of freshly passaged M.V. virus into the right sciatic nerve; in accordance with the suggestion first made by Fairbrother and Hurst[2] the nerve fibers were traumatized with the needle for the purpose of exposing some of the axis cylinders. The 4 animals promptly developed typical paralytic poliomyelitis with no indication that the absence of vitamin D deficiency in any way influenced the course of the infection.

For the next tests 4 apparently healthy rhesus monkeys were selected at random from a group of animals just arrived (November, 1940) from India. In 2 of these (3-40 and 3-42) roentgenograms of the long bones showed evidence of varying severity of rickets and scurvy. The concentration of inorganic P in the whole blood of 3 (3-39, 3-40, 3-42) was low and the concentration of organic acid-soluble P in the cells of one (3-39) was remarkably low. The borderline low normal concentrations of organic acid-soluble P in the cells of 2 (3-40 and 3-42) with moderately low concentrations of inorganic P, were considered to be compatible with mild rickets either in the course of development or of healing. Monkeys 3-39 and 3-41 were given 5,000 units of crystalline vitamin D in propylene glycol (drisdol, 20 drops) by mouth daily for 2 weeks before inoculation of the virus. On the 12th day the concentrations of inorganic P in the whole blood and of organic acid-soluble P in the cells were found to be greatly increased. Such increases in concentration of organic acid-soluble P to above normal have been found in the blood cells of rachitic rats after the administration of healing doses of vitamin D.[11] When virus was similarly injected into the right sciatic nerve of these 4 monkeys they all promptly developed poliomyelitis, and the course of the disease in the 2 vitamin D-deficient animals (3-40 and 3-42) did not differ significantly from that in monkeys without this deficiency.

Discussion. The results of this study indicate that vitamin D deficiency is not a necessary prerequisite for the spread of poliomyelitis virus along peripheral nerves. It may be of interest to note that Harrison and Woolpert[8] as well as Toomey[9] used glycerinated M.V. virus in their unsuccessful intrasciatic infection experiments while we used freshly passaged virus. A lower concentration of virus in the inoculum may, therefore, have been a factor in their experiments. Dr. Toomey has recently informed us that when he used our strain of virus and our procedure of intrasciatic inoculation, his monkeys regularly developed poliomyelitis regardless of whether or not they were deficient in vitamin D.

Summary. Poliomyelitis developed in all of 8 rhesus monkeys following the injection of freshly passaged "M.V." virus into their sciatic nerves. In 6 of these monkeys roentgenologic examinations of the long bones and determinations of inorganic and organic acid-soluble phosphorus in the blood just before inoculation gave no evidence of vitamin D deficiency. Indications of varying degrees of this deficiency were present in the other two animals.

There was no evidence that the capacity of poliomyelitis virus to invade the central nervous system along peripheral nerves depended on vitamin D deficiency.

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Simultaneous Inoculation of Variola and Vaccinia Viruses in Embryonated Eggs.

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In connection with some observations on the stability of variola virus it became of interest to determine the behavior of a small amount of vaccinia virus in the presence of a large amount of variola virus on inoculation in embryonated eggs. The relation of the ensuing findings to the problem of mutability as it pertains to variola virus will be considered elsewhere. The results are reported here simply as one example of the activity of 2 infective agents inoculated simultaneously in fertile eggs, a phase of that method on which there appears to be little data.

Two bacteriologically sterile strains of variola virus (Minnesota and Chinese) which had been carried through 202 and 38 transfers, respectively, in embryonated eggs were used, together with a single strain of vaccinia virus (New York City Board of Health) maintained by occasional egg transfer. The 2 variola strains react identically in 10-day fertile hen's eggs following implantation on the retracted chorioallantoic membrane. By the 3rd day a 10% membrane suspension of either strain produces a thickened confluent area at the site of inoculation but does not affect the embryo. The strain of vaccinia virus behaves very differently, and the 2 virus species may generally be identified by inspection. Eggs inoculated with this strain of vaccinia show a much thinner area of reaction in the membrane by the 3rd day, the result of rapid cell degeneration, and usually a dead embryo.

Saline suspensions were prepared containing variola virus diluted 10^1 and vaccinia virus diluted 10^6, the dilution figure being based on the wet weight of small membrane sections removed aseptically on the 3rd day from 10-day hen's eggs inoculated with the respective viruses. Prior to dilution the membrane sections were finely ground in glass tissue grinders. Similar dilutions, serving as activity controls, were also set up with each virus alone. Two or 3 embryonated eggs were then inoculated with approximately 0.5 cc of the virus mixtures and the individual suspensions. After incubation at 37°C for 3 days the inoculated eggs were opened for examination, and approximately 10% suspensions prepared from the membranes. Three to 5 successive egg transfers were subsequently made with each series.

The detailed results of one experiment are as follows: Two embryonated eggs opened on the 3rd day after inoculation with a mixture containing Chinese variola virus diluted 10^1 and vaccinia virus diluted 10^6 showed active embryos and the thickened membrane characteristic of variola, an identical reaction being obtained with the 10^1 dilution of variola virus alone. Eggs inoculated with the 10^6 dilution of vaccinia virus alone also showed active embryos with scattered discrete foci in the membrane, indicative of a highly diluted suspension of that virus. A 2nd transfer was made with each series, using 10% membrane suspensions. In the eggs inoculated with the virus mixture the embryos were inactive and the membranes moderately thickened, suggestive of an intermediate reaction. The eggs inoculated with variola virus alone showed the customary thickened membrane and active embryos; whereas in those inoculated with vaccinia virus alone the membrane was thin and the embryos dead, the characteristic reaction of this virus in low dilution. In the 3rd passage the individual virus suspensions behaved in the usual manner, but the mixture resulted in death of the embryos and thinning of the membrane at the site of inoculation, the typical manifestations of vaccinia virus alone.

Essentially the same results were obtained in 2 additional experiments using similar mixtures of vaccinia virus and the Chinese strain of variola, the former being masked in the first transfer but predominating by the 3rd. The procedure was repeated with mixtures of the same vaccinia virus and the Minnesota strain of variola. In the first test there was no apparent development of the vaccinia virus through the 5th transfer although it was demonstrable in the 10^6 dilution prior to mixing. In 2 subsequent tests, however, the preceding results were duplicated, vaccinia virus multiplying in the presence of a high concentration of variola virus and predominating by the 3rd transfer. It may be added that in the long series of egg inoculations made with the Minnesota strain of variola virus, since it was isolated in 1938, its behavior in embryonated eggs has remained constant with no departure in the direction of vaccinia virus.

Egg membranes from the 3rd transfer of the Chinese variola-vaccinia virus mixture and the 4th transfer of the mixture with the Minnesota strain were tested in dilutions through 10^7 by the inoculation of scarified areas in the skin of susceptible rabbits. A graded response typical of vaccinia virus was obtained through a dilution of 10^6. The corresponding suspensions of the two variola strains alone behaved in the customary way, producing no macroscopic reaction in the skin of inoculated rabbits. Cutaneous inoculation of the monkey to determine the presence of variola virus in membranes inoculated with the virus mixtures was not carried out.

Summary. Mixtures of variola virus diluted 10^1 and vaccinia virus diluted 10^6 were inoculated in embryonated eggs and followed by 3 or more successive transfers. In 5 of the 6 tests the highly diluted vaccinia virus was masked in the 1st transfer but subsequently multiplied and predominated by the 3rd. Two of the transferred mixtures on inoculation in rabbits produced a typical vaccinial reaction in dilutions through 10^6.

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Biotin and Prevention of Dermatitis in Turkey Poults.*

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During attempts to determine the riboflavin requirement of poults, in which both simplified and commercial type rations were used, dermatitis occurred regardless of the riboflavin intake. Jukes[1]

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[1] Jukes, T. H., *Poultry Sci.*, 1938, **17**, 227.

reported that riboflavin prevented dermatitis in poults. Recently, Hegsted, *et al.*,[2] reported a dermatitis in chicks distinct from pantothenic acid deficiency and apparently unrelated to riboflavin. Since the dermatitis observed by us in turkeys appeared to be similar to that described by Hegsted, *et al.*, the essential rôle of biotin in turkey nutrition was investigated.

The basal ration had the following percentage composition: corn starch, 32.8; purified casein, 30; purified wheat bran, 20; polished rice, 5; soybean oil, 5; cod liver oil (400 D), 1; wheat germ oil, 2; mineral mixture, 4; choline, 0.2; thiamin, 0.0005. and pyridoxin, 0.0005. Day-old Bronze turkey poults were used throughout the experiment. They were kept in electrically heated brooders, equipped with hardware cloth floors. The experimental ration and water were always available.

The results recorded in Table I are typical of those secured in experiments involving more than 1000 poults. Incipient lesions ap-

TABLE I.
Dermatitis Preventive Action of Supplements.

Supplements		Results at 4 weeks of age			Disorders		
Riboflavin, %	Concentrates	Poults No.	Mortality %	Wt, g	Eyes %	Feet %	Beak %
0	None	6	83	82	83	34	83
.0002	”	32	78	100	41	25	53
.0005	”	18	72	203	22	44	67
”	5% feeding cane molasses	21	71	181	48	43	62
”	.014% Ca-pantothenate	18	39	210	55	78	83
”	.4% nicotinic acid	12	58	115	0	50	50
”	8% filtrate from peptic digest of liver	12	42	190	0	0	0
”	10% norite adsorbate of filtrate from peptic digest of liver	12	87	202	0	0	0
”	5% water extr. of liver (4080)*	6	100	—	33	100	100
”	4% alcohol extr. of liver (4303)*	6	17	296	0	33	50
”	10% liver residue (4081)*	5	0	392	0	0	0
”	10% desiccated liver	8	0	417	0	0	0
”	3.2% R-S concentrate†	12	87	200	25	75	75
”	5% yeast residue†	26	4	330	0	0	0
	5% ” ”	22	36	136	0	0	0
”	5% dried yeast‡	20	5	381	0	0	0
”	40 rat units biotin daily per poult	10	10	180§	0	0	0
”	Control for biotin supplemented group	9	44	182§	11	89	89

*Prepared by methods described by Hogan, A. G., Richardson, L. R., Patrick, H., and Kempster, H. L., *J. Nutr.*, 1941, **21**, 327.

†Prepared as outlined by Schumacher, A. E., and Heuser, G. F., *Poultry Sci.*, 1940, **29**, 315.

‡Strain-S brewer's yeast generously furnished through the courtesy of Dr. K. L. Cartwright of Anheuser-Busch & Co.

§These body weights were taken at 21 days.

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[2] Hegsted, D. Mark, Oleson, J. J., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Nutr.*, 1940, **20**, 599.

peared at 2 to 3 weeks of age. The areas between the toes, on the bottoms of the feet and at the proximal-dorsal portion of the upper beak showed definite lesions. The feet became completely encrusted and hemorrhagic cracks appeared as the manifestation of the deficiency progressed. The toes, in severe cases, became necrotic and sloughed off while the shank became calloused. The eyes became encrusted and eventually closed due apparently to an exudate. It is felt that this eye disorder is not necessarily an accompanying feature of the dermatitis since its severity did not parallel the incidence of dermatitis on the feet and beak.

Crystalline riboflavin, varying from none to 0.0005%, did not protect the turkeys from dermatitis. Calcium pantothenate, nicotinic acid, cane molasses, the water-soluble fractions of dried beef liver and dried brewer's yeast, and the alcohol-soluble fraction of dried beef liver were also ineffective. The residues from liver and yeast gave complete protection.

György, *et al.*,[3] stated that biotin could not be removed from liver or yeast by ordinary methods of solvent extraction but could be removed by peptic digestion. Accordingly liver was digested with pepsin and the filtrate concentrated *in vacuo*. The peptic filtrate and a norite adsorbate of the peptic filtrate were both active. This suggested that biotin was probably the active factor present in the liver and yeast residues. To confirm this point a biotin concentrate (S.M.A. 5000) reportedly free of other members of the B complex, was fed daily by pipette at a level of 40 rat units per day. Complete protection from dermatitis resulted while a group receiving the same ration, but without biotin, developed severe dermatitis in 89% of the poults.

Poults receiving the basal ration supplemented with 5% yeast residue grew slowly and did not develop dermatitis. Poults receiving the basal ration plus 5% yeast residue and 0.0005% riboflavin grew rapidly without developing dermatitis.

Under the conditions of this experiment riboflavin did not prevent dermatitis in turkey poults, while biotin at a level of 40 rat units per day was effective. Other concentrates used in feeding trials, reported in Table I, also possessed the dermatitis-preventing properties ascribed to biotin.

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[3] György, P., Kuhn, R., and Lederer, E., *J. Biol. Chem.*, 1939, **131**, 745.

13356 P

The Anti-Perosis Complex.*

L. R. Richardson and A. G. Hogan.

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The rôle of manganese in preventing perosis in chicks was established[1] some time ago, and more recently it was demonstrated by Hogan, Richardson, and Patrick[2] that an organic factor is equally essential. Both are required to prevent the disease. This organic nutrient is present in a 95% alcohol extract of dried liver but it is absent from the water extract which was prepared after the alcohol extraction. Jukes[3] identified choline with the antiperotic agent. Hogan, *et al.*,[4] reported that choline protects chicks against perosis under certain conditions but in their experience it was not the only organic substance required. This report is to describe a type of perosis which results from a deficiency of a third, as yet unidentified, nutrient.

Day-old, single-comb White Leghorn chicks were used as experimental animals. The basal ration has the following composition: Casein 35, starch 31, cellulose 3, salt mixture[5] 4, lard 17, acid hydrolyzed liver residue 10. Each 100 g of the ration was supplemented with 6000 I.U. of vitamin A, 850 I.U. vitamin D, 0.8 mg thiamin chloride, 1.6 mg riboflavin, 1.2 mg pyridoxine, 2.0 mg calcium pantothenate, 1.0 mg 2-methyl-1,4-naphthoquinone, and 0.8 mg of alpha tocopherol. The ration contained 0.025% manganese and the crude vitamin carriers were substituted for an equal weight of starch.

The water extract of dried beef liver, No. 4080, which was described by Hogan, *et al.*,[4] was used as a source of the unrecognized vitamins required by the chick. This extract was treated with fuller's earth at a pH of 1.0, and the adsorbate was then eluted with 0.2 N ammonia. Choline was supplied to all chicks except those in

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* Contribution from the Department of Agricultural Chemistry of the Missouri Agricultural Experiment Station. Journal Series No. 780.

[1] Wilgus, H. S., Jr., Norris, L. C., and Heuser, G. F., *J. Nutrition*, 1937, **14**, 155.

[2] Hogan, A. G., Richardson, L. R., and Patrick, H., *Proc. Am. Inst. Nutr.*, p. 12; *J. Nutrition*, 1940, **19**.

[3] Jukes, T. H., *J. Nutrition*, 1940, **20**, 445.

[4] Hogan, A. G., Richardson, L. R., Patrick, H., and Kempster, H. L., *J. Nutrition*, 1941, **21**, 327.

[5] Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition*, 1937, **14**, 273.

Group I. The other supplements were added to the basal ration as follows:

Group I and II, water extract of liver, 6%.

Group III, filtrate after fuller's earth adsorption of the water extract of liver at pH 1.0, 6%.

Group IV, inositol 100 mg, nicotinic acid 10 mg, and p-aminobenzoic acid 30 mg, per 100 g of the basal ration.

Group V, fuller's earth adsorbate of the water extract of liver at pH of 1.0, 6%.

Group VI, a 0.2 N ammonia eluate of the fuller's earth adsorbate, 1%.

These data are summarized in Table I.

TABLE I.
Effect of Choline and of Liver Extract on Perosis.*

				Perosis		
Group	Supplement	Choline, %	No. of chicks	Incidence, %	Index†	Mortality, %
I	Water extract of liver	0.0	11	100	33.0	0.0
II	,, ,, ,, ,,	0.1-0.4	55	3.6	1.0	2.0
III	Filtrate after fuller's earth adsorption	0.4	12	91.6	30.0	19.6
		0.4-0.6‡	12	83.3	25.9	41.6
IV	Inositol, nicotinic acid and p-aminobenzoic acid	0.6	6	100.0	34.0	0.0
V	Fuller's earth adsorbate at pH of 1.0	0.1-0.6	55	5.4	1.8	0.0
		0.4‡	9	11.1	2.1	11.1
VI	0.2 N ammonia eluate of fuller's earth adsorbate	0.4	22	8.6	1.6	4.3
		0.4‡	5	0.0	0.0	20.0

*Experimental period 6 weeks.

†Calculated by the method of Wilgus, Norris and Heuser.[6]

‡An equal quantity of gelatin was substituted for the acid hydrolyzed liver residue and each chick received 3 γ of biotin per day, SMA Concentrate 200.

Every chick which received the water extract of liver without choline (Group I) developed a severe perosis. The perosis which developed on this type of ration was prevented almost completely by including 0.1 to 0.4% of choline in the diet (Group II). When the water extract of liver was replaced by the filtrate after fuller's earth adsorption (Group III), 0.4 to 0.6% of choline did not prevent the occurrence of severe perosis. The addition of inositol, nicotinic acid, and p-aminobenzoic acid (Group IV) or 3 gamma of biotin per chick per day (Group IIIa) were ineffective.

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[6] Wilgus, H. S., Jr., Norris, L. C., and Heuser, G. F., *Poultry Sci.*, 1937, **16**, 232.

These data show that at least one nutrient is required, in addition to choline and manganese, to prevent perosis in chicks. Furthermore, this substance is not identical with any of the recognized vitamins. The fuller's earth adsorbate (Group V) and a 0.2 N ammonia eluate of the fuller's earth adsorbate (Group VI) are fair sources of the nutrient.

The perosis due to a deficiency of the unidentified factor is similar to that produced by a deficiency of manganese or choline, but is usually less severe. If it were possible to supply all other essential unrecognized vitamins in a concentrated form, the chicks probably would grow faster, and presumably they would develop more severe symptoms.

13357

Relationship Between Anesthetic Potency and Physical Properties.*

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A new series of cyclopropyl aliphatic ethers has been synthesized in this laboratory. The first two members of this series, cyclopropyl methyl ether (cyprome ether) and cyclopropyl ethyl ether (cypreth ether) have been shown to be useful anesthetics.[1, 2, 3] In comparative studies with these new agents it became necessary to study the physical properties of these compounds to determine their value in predicting anesthetic potency. The literature available on the oil/water coefficients of the generally used anesthetics is confusing and data are not concordant. The authors believe that this is due to: first, the technical difficulties inherent to the determination; second,

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* The material contained in this paper is part of a thesis submitted by Nellie M. Cone to the Faculty of the Graduate School of the University of Maryland in partial fulfillment of the requirements for the degree of Master of Science.

The expense of this investigation was defrayed in part by a grant from the Ohio Chemical and Manufacturing Company of Cleveland, Ohio.

[1] Krantz, J. C., Jr., Carr, C. J., Forman, S. E., and Evans, W. E., Jr., *J. Pharm. and Exp. Therap.*, 1940, **96**, 207.

[2] Black, C., Shannon, G. E., and Krantz, J. C., Jr., *Anesthesiology*, 1940, **1**, 274.

[3] Krantz, J. C., Jr., Carr, C. J., Forman, S. E., Evans, W. E., Jr., and Wollenweber, H., *J. Pharm. and Exp. Therap.*, 1941, **72**, 233.

the use of oils containing free fatty acids, and third, the employing of analytical procedures which lack precision. Accordingly, the field was restudied and more dependable comparative data obtained.

Oil/Water Coefficients. Corn oil was selected as the fat of choice as it is available with a titer of less than 0.03% fatty acids. Fifty cubic centimeters of the oil was weighed in a bottle of about 200 cc capacity. A volume of the anesthetic agent was added, calculated to make the oil volume 0.1 Molar with respect to the solute. Having stoppered the bottle with a tightly-fitting, tin-foil covered stopper, the amount of anesthetic introduced was determined by weighing. An equal volume of water was added and the bottle agitated vigorously for 1 hour in a constant temperature room at 25° ± 0.5°. Most of the oil was withdrawn by gentle suction through a pipette and exactly 1 cc of the perfectly clear aqueous layer removed in a chilled pipette for analysis. With certain minor modifications the dichromate method of Andrews, *et al.*,[4] for the quantitative determination of ether was applicable to the determination of these compounds. The results are set forth in Table I.

TABLE I.
Oil/Water Coefficients of Anesthetics.

Compound	No. of determinations	Oil/water coefficient	Probable error ±
Ethyl ether	10	4.1	.8
Cyprome ether	9	5.0	.4
Cypreth ether	7	16.3	.7
Allyl methyl ether	10	4.1	.5

Despite the precautions observed and the tediousness of the determination the probable error in a series of analyses is rather high. It occurred to the authors to attempt again, as many previous investigators have done, to correlate some definite physical property with anesthetic potency.

In the series of anesthetic agents studied we have found the simple determination of the water solubility of the compound to bear a striking relationship to the biological potency. This datum appears to be a more faithful physical index than the oil/water coefficient and the simplicity of determination bespeaks its general applicability. Our determinations were carried out in 100 cc "Cassia Flasks." One hundred cubic centimeters of water was introduced and an exact volume of the anesthetic agent in excess of its water solubility was added. The flask was agitated vigorously for 2 hours and after complete separation, the volume of the undissolved agent was measured in the graduated neck of the flask. We have expressed our data most significantly by plotting the reciprocal of the solubility (1/cc in 100 cc) against the logarithm of the number of moles per kg which we determined were required to produce respiratory arrest in the dog. Fig. 1 shows these data.

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[4] Andrews, E., Potter, R. M., Friedmann, T. E., and Livingstone, H. M., *J. Lab. and Clin. Med.*, 1940, **25**, 966.

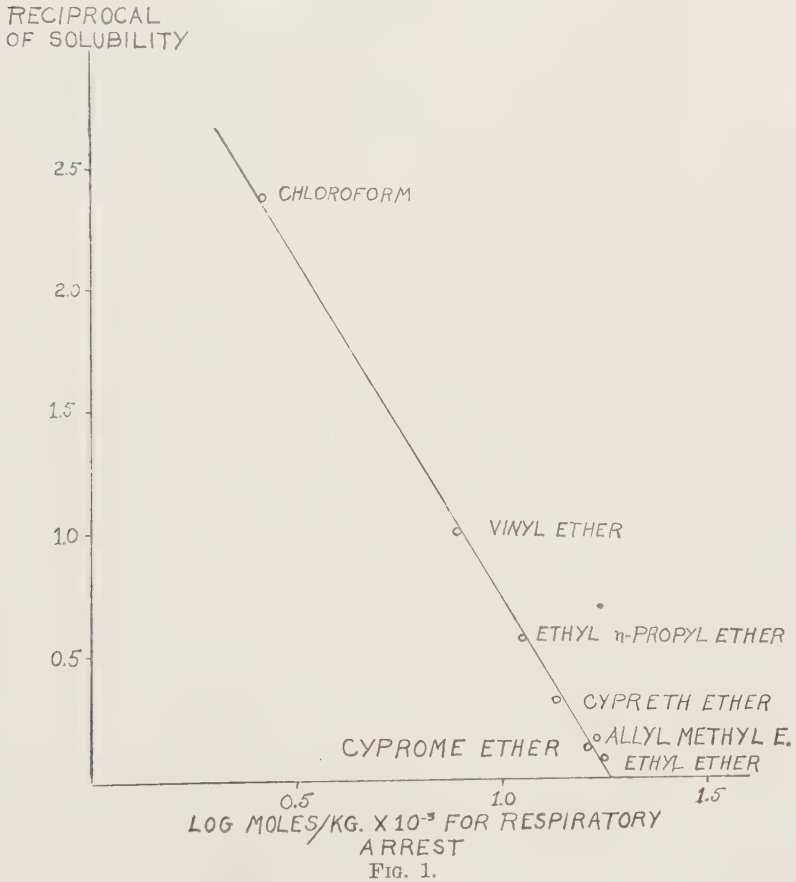

FIG. 1.

Summary. In a series of new anesthetics and also among certain of those agents widely employed today, the authors have found the insolubility of the compound in water a faithful criterion of anesthetic potency.

13358

Absorption of Estradiol and its Esters from Subcutaneously Implanted Tablets in the Guinea Pig.*

ALEXANDER LIPSCHÜTZ AND LUIS VARGAS, JR.

From the Department of Experimental Medicine, National Health Service of the Republic of Chile, Santiago.

Deanesly and Parkes made use of their ingenious method of subcutaneously implanted tablets to study the question of the rate of hormone absorption. Emmens[1] has recently communicated important results on the comparative rate of absorption of estrogens and androgens and their esters in the rat.

In the course of our work on the fibromatogenic action of a prolonged treatment with estrogens in the guinea pig, we have applied the method of Deanesly and Parkes with most satisfactory results.[2] In this way, we also became interested in the rate of absorption of different hormones. In the present paper, we shall give the results obtained in 52 female guinea pigs with 3 estrogens: free estradiol, dipropionate of estradiol and 17-caprylate of estradiol. Our results (Table I) which confirm those of Emmens, present new relevant details and render it possible to compare the rate of absorption in two different species.

The range of weight of the disc-shaped tablets, or their fragments, was the same in all 3 groups, and range of surface was very likely the same. This is important because absorption is related to the surface. There was considerable scattering of percentage of absorption in each of these three groups (fifth row of Table I and Fig. 1), but it is remarkable that the range of scattering of percentage of absorption coincided with the range of weight of tablets in the respective group (fourth row of Table I, figures in parentheses). The smaller or the thinner a tablet, the greater the surface in relation to the weight and the greater also the percentage of absorption.

The average absorption of estradiol was 20% in 73 days, or 0.28% per day (sixth row of Table I). The caprylate tablets lost 6.1% in 74 days or 0.08% per day. The corresponding figures of Emmens

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* This investigation has been aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research and the Rockefeller Foundation. Grants administered by Professor A. Lipschütz. All estrogens were generously supplied by Dr. Carl Miescher of Ciba Pharmaceutical Products, Inc.

[1] Emmens, C. W., *Endocrinology*, 1941, **28**, 633.

[2] Lipschütz, A., and Vargas, L., Jr., *The Lancet*, 1939, **1**, 1313.

TABLE I.

(1)	(2)	(3)	(4)	(5)		(6)			
							Avg. absorption calculated as *free* estradiol		
Group	No. of animals	Duration of exp. days	Avg wt of tablet, mg	Avg absorption		Avg absorption per day, %	Total, mg	Per day, γ	Per day per mm², γ
				mg	%				
Free estradiol (series XXVII, No. 21-37)	17 (310-740 g)*	73	21.2 (12-34)*	4.25 (1.7-6.9)*	20.2 (10-38)*	.28	4.25	58	1.2-1.4
Dipropionate of estradiol (series XXX, No. 1-21)	16 (315-855 g)	70	16.9 (12-28)	5.49 (3.2-7.6)	32.5 (23-46)	.46	3.93	56	1.1-1.6
17-caprylate of estradiol (series XXXI, No. 2-25)	19 (430-725 g)	74	19.2 (14-27)	1.21 (0.8-1.7)	6.1 (4.6-7.8)	.08	0.83	11	0.2

*Figures in parentheses indicate range.

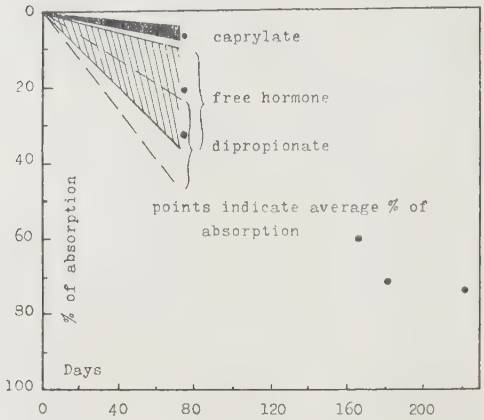

FIG. 1.

Percentage of absorption from subcutaneously implanted tablets of estradiol, and dipropionate and 17-caprylate of estradiol in 52 castrate female guinea pigs. Scattering curves of percentage of absorption and average percentage of absorption in the course of 70 to 74 days. Percentage absorption of dipropionate in three females in the course of 168 to 222 days reaching 74%.

in the rat were 0.26 and 0.08% per day in 91 days. Though there were differences in weight and shape of the tablets used in guinea pigs and rats, the rate of absorption coincided in the two species.

The average percentage of absorption of the dipropionic ester was 32% in 70 days or 0.46% per day in our guinea pigs, and 0.3% in the rat.[1] Though there is a considerable gap between these two figures, both indicate that the dipropionic ester of estradiol behaved differently as to its rate of absorption from the caprylate and, as shown by Emmens, also from the remaining known esters.

The tablets of estradiol and its dipropionate were disc-shaped. The diameter was 7 mm, and thickness was about 1 mm. A central notch allowed for easy breakage into 2 more or less equal halves. The weight of one half was 25 mg, and the surface was somewhat less than 50 mm^2. Since several fragments were smaller or thinner (average weight only 21 and 17 mg respectively instead of 25, see Table I), the average surface must have been less than 50 mm^2 but certainly no less than 42 and 34 mm^2 respectively (2 mm^2 per 1 mg). The diameter of tablets of caprylate was 5.25 mm and the thickness about 1 mm. The surface of a tablet of 20 mg was somewhat more than 50 mm^2. In the last row of Table I, absorption is calculated per square millimeter and day; even when allowance was made for error in the calculation of the average surface (42 to 50 mm^2 for estradiol, 34 to 50 mm^2 for dipropionate) the results were not altered significantly. An average of 1.2 to 1.4 γ of estradiol was absorbed daily per square millimeter of a tablet of free estradiol. A similar quantity of estradiol was available daily from one square millimeter of a tablet of dipropionate (1.1 to 1.6 γ), *i. e.,* the rate of absorption was, in our experiments, so greatly enhanced by combination with propionic acid that a tablet of dipropionate gave up per day and per square millimeter the same number of molecules of estradiol as a tablet of the free hormone. On the contrary, five to six times less was absorbed daily per square millimeter of a tablet of caprylate. This would explain why dipropionate of estradiol is so similar to estradiol in the estrous test in the rat whereas other esters are much less estrogenic.[3]

Summary. The rate of absorption of free estradiol, and dipropionate and caprylate of estradiol from subcutaneously implanted tablets has been studied in the *guinea pig.* The average percentage absorption per day was very similar to that found by Emmens in the *rat.* Average percentage absorption from 17-caprylate tablets was 3 times less than that from tablets of free estradiol. On the contrary, average percentage of absorption from dipropionate tablets was greater than from estradiol tablets. Combination of estradiol with two molecules of propionic acid so greatly enhanced the percentage absorption in the guinea pig that the number of molecules of estradiol available from one square millimeter of a dipropionate tablet became similar to that available from one square millimeter of a tablet of the free hormone.

13359

Conjunctive Tumorigenesis Elicited by Different Artificial Estrogens.*†

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From the Department of Experimental Medicine, National Health Service of the Republic of Chile, Santiago.

As with *natural* follicular hormones, free or esterified, a prolonged treatment with an *artificial* estrogen, such as stilbestrol, will induce

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[3] Miescher, K., Scholz, C., and Tschopp, E., *Biochem. J.*, 1938, **32**, 725.

* This work has been aided by grants from the Jane Coffin Childs Memorial Fund for Medical Research and from the Rockefeller Foundation. Grants administered by Prof. A. Lipschütz.

† Medical Clinic of the Faculty of Medicine.

uterine and extragenital subserous fibroids in the abdominal cavity.[1] No difference as yet has been found as to the localization and microscopical structure of fibroids induced by either of these estrogens.[2] On the other hand, stilbestrol has been shown to be more tumorigenic than free natural hormones (α-estradiol and estrone) and less tumorigenic than the esters of estradiol; with greater doses the tumorigenic action of stilbestrol was equal to that of similar doses of the esters of estradiol.[1] The greater tumorigenic action of stilbestrol, when compared with free estradiol, is probably due to the greater resistance against inactivation which the artificial estrogens offer in the body.[3] These properties of stilbestrol raised the question of the tumorigenic power and behavior of other artificial estrogens and similarly, the increased tumorigenic power of esterified natural female hormones led to a study of the effect of esterification on the fibromatogenic behavior of an artificial estrogen.

Experiments with Hexestrol. Forty-nine castrate female guinea pigs (290 to 630 g) were given thrice weekly subcutaneous injections of hexestrol[4] in olive oil, in the course of 3 months. Quantities injected varied between 0.05 and 80 γ per injection. The vagina remained open, and normal uterine weights were reached or surpassed with 0.3 to 0.6 γ per injection. Small nodules (tumoral seed) appeared on the spleen, stomach, diaphragm and abdominal wall, even with minute quantities of hexestrol (0.05 to 0.6 γ per injection). The microscopical structure of these nodules was typical of those obtained with other estrogens.[2] The tumoral seed became more conspicuous with 1 to 5 γ per injection. With 10 γ per injection, uterine fibroids of "class 1", according to our classification,[5] were elicited. With higher doses, a tumorigenic action, similar to that with the esters of estradiol, was produced (Fig. 1). A comparative survey of the results with hexestrol and other estrogens is given in Table I.

Notwithstanding the great individual variations noted with experimental fibroids, the table gives full evidence that not only is hexestrol more tumorigenic than the natural free hormones but also

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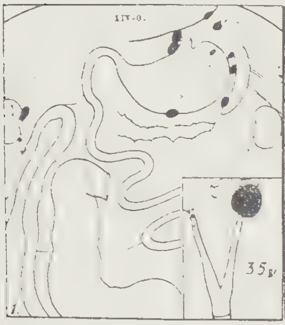
[1] Lipschütz, A., and Vargas, L., Jr., *The Lancet*, 1940, **1**, 541.

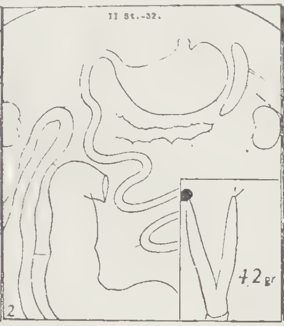
[2] Lipschütz, A., and Vargas, L., Jr., *Cancer Research*, 1941, **1**, 236.

[3] Stroud, S. W., *J. Endocrinol.*, 1939, **1**, 201; Dingemanse, E., and Tsylowitz, R., *Endocrinology*, 1941, **28**, 450.

[4] Campbell, N. R., Dodds, E. C., and Lawson, W., *Nature*, 1938, **142**, 1121. We are indebted to Prof. E. C. Dodds for a generous supply of hexestrol.

[5] Lipschütz, A., and Vargas, L., Jr., *C. R. Soc. Biol.* (Paris), 1939, **131**, 27. Additional data, see Lipschütz, A., Bellolio, P., Chaume, J., and Vargas, L., Jr., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 164.





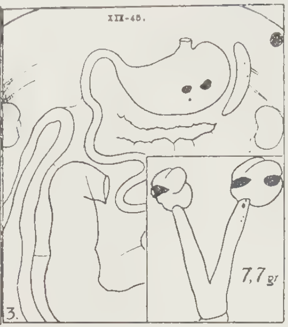


FIG. 1.

Castrate female guinea pig (355 g when castrated). 40 injections of 40 γ of hexestrol in the course of 88 days. Small uterine fibroid, large apical tumor of left uterine horn and multiple large abdominal fibroids. × 0.3. (XIV, 8).

FIG. 2.

Castrate female guinea pig (250 g when castrated). 47 injections of 40 γ of stilbestrol in the course of 114 days. Apical tumor of right uterine horn. Small subserous uterine and extragenital fibroids. × 0.3. (II. St. 32.)

FIG. 3.

Castrate female guinea pig (430 g when castrated). 48 injections of 20 γ of stilbestrol given as dipropionate in the course of 111 days. Small subserous uterine fibroids. Fibroids in the wall of tubar cysts. Large extragenital tumors. × 0.3. (XIX, 45.)

The tumoral effect in Fig. 1, 2 and 3 was classified as follows:

	Subserous or parametrial uterine tumor, class	Apical tumor, class	Tumor of dig. tract and abdom. wall, class	Splenic tumor, class	Total tumoral effect*
Fig. 1	0.5	3	3	2	8.5
” 2	0.5	2	0	0.5	3.0
” 3	1	2	2	0.5	5.5

*The sum of the 4 numerical classes.

more tumorigenic than stilbestrol. Hexestrol attains the tumorigenic action of the 2 esters of estradiol, even with 40 γ per injection. It is also remarkable that the initial conjunctive reaction (tumoral seed) can be found, though only exceptionally, with minute quantities of hexestrol coincident with, or even below, the hysterotrophic dose necessary to maintain a normal uterine weight. There was one case which, having received only 0.3 γ of hexestrol per injection, developed a small intramural fibroid in the uterine submucosa. Similar conjunctive reactions towards such minute quantities have as yet not been seen in this department with other estrogens, natural, esterified or artificial.

Experiments with Dipropionate of Stilbestrol.[‡] All quantitative

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[‡] Dipropionate of stilbestrol was at our disposition only in an oily solution manufactured by Messers Bayer (local branch). The solution (B-Cyren, "fuerte") contains 2.5 mg of dipropionate per ml. One mg of stilbestrol is contained in 1.42 mg of dipropionate.

TABLE I.
454 Castrate Female Guinea Pigs Injected with Natural and Artificial Estrogens, Thrice Weekly, in the Course of 3 Months.

Quantity per injection, γ	Estradiol and estrone 91 animals*	Stilbestrol 48 animals†	Hexestrol 56 animals‡	17-benz. and 3-17-prop. of estradiol 218 animals§	Diprop. of stilbestrol 41 animals
.05-.6	0	0	±	0	0
1	0	0	±	±	•
2	0	•	•	•	•
3-4	0	±	±	•	±
5	0	±	±	1	1
10	0	+	1	1	1
20	0	•	2	5	5
40	0	1	4	4	4
80	0	5	5	5	5
100	±	5	•	•	•
250-300	+	4	•	6	•
400-500	4	5	•	•	•

*Rodríguez, F., *Public Med. Exp.* (Chile), 1940.

†See [1] and unpublished results.

‡Results of Palma, J., *Public Med. Exp.* (Chile), 1940, No. 6 included.

§Iglesias, R., *Public Med. Exp.* (Chile), 1938; Bellolio, P., *ibid.*, 1939; Murillo, R., *ibid.*, 1940; Lipschütz *et al.*, PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 164; and unpublished work. Different animals treated for less or more than 3 months also are included in this column.

Explanation of signs:

0 = no visible macroscopical reaction.
± = only initial reaction (fibrous strands; tumoral seed).
+ = only small fibroids not reaching "class 1."
1, 2 etc. = fibroids of class 1 or more; the *average* "total tumoral effect" is given.
• = no data available.

and timing conditions were identical with those in the foregoing experiments. Table I indicates that the tumorigenic power of stilbestrol is enhanced by esterification (see also Figs. 2 and 3). With doses of 10 to 80 γ per injection, the dipropionate of stilbestrol is probably equal to the highly tumorigenic 17-caprylate of estradiol.[6] Dodds, *et al.*,[7] state that esterification of stilbestrol favors a protracted action. According to our former results, estrogens acquire tumorigenic faculties when a stable threshold folliculinaemia is maintained for a sufficiently long time,[8] and this is probably facilitated by esterification.

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[6] Lipschütz, A., Vargas, L., Jr., Baeza-Rosales, H., and Baeza-Herrera, H., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 76.

[7] Dodds, E. C., Goldberg, L., Lawson, W., and Robinson, R., *Nature*, 1938, **211**, 142.

[8] Lipschütz, A., *Libro de Homenagem Prof. A.e.M. Ozorio de Almeida*, Rio de Janeiro, 1939, p. 413; Lipschütz, A., and Vargas, L., Jr., *C. R. Soc. Biol.* (Paris), 1939, **130**, 9; Lipschütz, A., Rodríguez, F., and Vargas, L., Jr., *Endocrinology*, 1941, **28**, 664.

Summary. Uterine and extrauterine fibroids can be induced in the guinea pig by a prolonged treatment with hexestrol. Like stilbestrol, hexestrol also is more tumorigenic than the free natural follicular hormones (estrone and estradiol). The tumorigenic power of hexestrol is even greater than that of stilbestrol. The tumorigenic power of stilbestrol is, like that of estradiol, greatly enhanced by esterification.

13360 P

Effects of Roentgen Irradiation upon Linear Rate of Flow in Cutaneous Lymphatics of Humans.

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The effects of irradiation upon the function of lymphatics is of clinical as well as experimental significance. Many clinicians believe that irradiation therapy can be used to obstruct lymphatics and thereby reduce the ease of metastasis of malignancy. Sufficient physiological observations necessary to evaluate the opinion are lacking. Teneff and Stoppani[1] employing thorium dioxide and India ink for visualization found evidence of changes in lymph circulation of the subcutaneous tissues of dogs and guinea pigs only when large doses of X-ray sufficient to produce destruction of cells were used. Hodes and Griffith[2] using the thorium dioxide method of Menville and Ané[3] failed to find any significant interference with lymph flow in the subcutaneous lymphatics even with doses as large as 2,200 r. Accordingly, the observations to be described were initiated in an attempt to learn some of the effects of roentgen irradiation upon the cutaneous lymphatics of humans.

Seven white adults (19 to 57 years of age) who were receiving roentgen therapy for various clinical states were selected as subjects. The linear rate of flow in the cutaneous lymphatics of 12 different skin areas were studied 45 times by the colloidal dye method of McMaster.[4] The subjects rested quietly for about 30 minutes in the

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[1] Teneff, S., and Stoppani, F., *Radiol. med.*, 1935, **22**, 768.

[2] Hodes, P. J., and Griffith, J. Q., *Radiology*, 1941, **37**, 203.

[3] Menville, L. J., and Ané, J. N., *J. A. M. A.*, 1932, **98**, 1796.

[4] McMaster, P. D., *J. Exp. Med.*, 1937, **65**, 347.

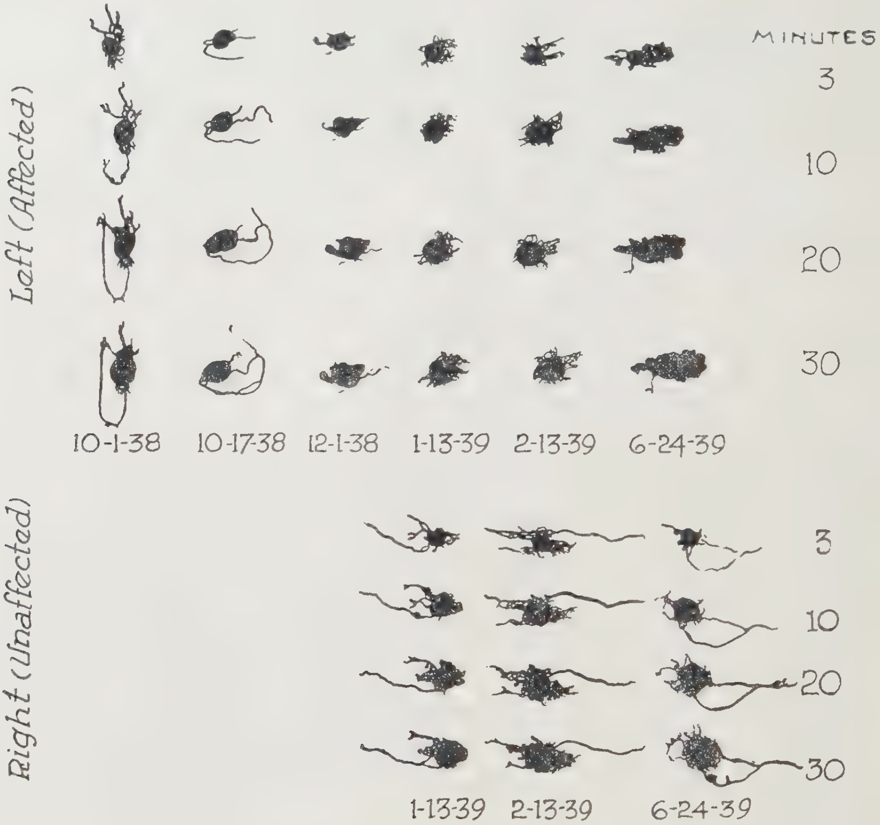

Fig. 1.

Tracings of the cutaneous lymphatics of the right and left side of the lower part of the abdomen of one subject. On the left or affected side, there was a first degree skin reaction produced by the irradiation (total dosage 1,187 r) while on the right side no visible reaction occurred (total dosage 370 r). The horizontal rows of tracings from above downward represent the visualized lymphatics for the respective 3-, 10-, 20-, 30-minute periods following injection of the dye, while the vertical rows represent the visualized lymphatics for the date indicated below.

supine or prone position, depending upon the area of skin studied, before the injections of the lymphatics were made. The visualized lymphatics were then traced on cellulose acetate sheets at intervals of 3, 10, 20, and 30 minutes following each injection, the tracings being kept for future analysis for linear rate of lymph flow. The injections were made just before and at varying periods after beginning therapy. When two areas were studied the injections were made within 90 seconds of each other. In order to control the amount of dye injected, a syringe previously described was employed.[5] All irradiated areas of skin extended well beyond the lymphatics visualized.

In the 10 different irradiated areas studied in the subjects who received doses varying from 220 r to 450 r the linear rates of lymph flow remained within normal variations. One subject received 370 r on the right side of the lower part of her abdomen and 1,187 r on a comparable area on the left. On the former side there were no detectable changes in the linear rate of lymph flow. Six weeks following the production of a first degree skin reaction on the left side there was a definite decrease in the rate of flow. During the course of 8 months the rate remained normal on the right side, while that on the left remained markedly reduced (Fig. 1). It was also observed that there was a great tendency for the dye to diffuse locally around the site of injection, rather than to form small streamers as occurs in normal skin.

Summary. The data tend to indicate that small doses of X-ray (220-450 r) to the skin of humans are not likely to disturb the linear flow of lymph in the cutaneous lymphatics, while large doses sufficient to produce a first degree skin reaction probably will reduce the rate of lymph flow.

13361 P

"Leg-Weakness" in Laying Hens on an Ascorbic Acid-Free (Vitamin C) Diet.*

T. A. Bell, G. H. Satterfield and F. W. Cook. (Introduced by E. V. McCollum.)

From the Departments of Chemistry and Poultry Science, North Carolina State College of Agriculture and Engineering, University of North Carolina, Raleigh, N.C.

Since 1919 many workers[1, 2, 3] have demonstrated the growth of chickens on a supposedly vitamin C-free diet. There are reports[4, 5] indicating the possibility of a vitamin C requirement for the chicken.

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[5] Burch, G. E., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 676.

* Submitted by T. A. Bell in partial fulfillment for the degree Master of Science.

[1] Hart, E. B., Steenbock, H., Lepkovsky, S., and Halpin, J. G., *J. Biol. Chem.*, 1925, **66**, 813.

[2] Plimmer, R. H. A., Rosendale, J. L., and Raymond, W. H., *Biochem. J.*, 1923, **17**, 787.

[3] Shorten, J. A., and Ray, C. B., *Biochem. J.*, 1921, **15**, 274.

[4] Holst, W. F., and Halbrook, E. R., *Science*, 1933, **77**, 354.

[5] Beaudette, F. R., *Hints to Poultrymen*, 1923, 12, No. 2, The N.J. Agricultural Experiment Station.

In the course of an experiment to ascertain the influence of injections of ascorbic acid on the ascorbic acid content of blood, eggs, feces, and certain organs of laying hens[6] a muscular weakness developed in the legs of 3 hens fed a vitamin C-free diet under laboratory conditions.

In this study 15 Barred Plymouth Rock hens, 3 groups of 6, 6, and 3, were fed the N. C. State College laying mash, scratch grain treated with 1% cod liver oil, and oyster shell.[7] Fresh water was supplied daily. Each new supply of mash and grain was tested for its vitamin C content using the indophenol titration method,[8] and all the samples were found to be devoid of vitamin C. The hens were confined to laying batteries in a room in a laboratory.

This study was started January 14, 1939, and was terminated June 13, 1939. Group A (test) hens were given injections of ascorbic acid throughout the experiment. The first week 2 injections of 100 mg of ascorbic acid in 2 ml of Ringer's solution[9] were made subcutaneously. Thereafter, for the next 9 weeks 50 mg per injection were used twice weekly, then 4 injections were made weekly in the final 9 weeks of the experiment. A total of 246 injections of ascorbic acid were given to the 6 hens in group A which remained normal throughout the experiment. The hens in the control groups B and C, with the exception of those that developed "leg-weakness", received no ascorbic acid. The hens that developed this "leg-weakness", B3, C1, and C2, had been on the experimental diet 62, 67, and 68 days respectively.

The morbid condition of each of these 3 hens was similar: weakness and loss of action of one or both legs, inappetence, somnolence, inanition with loss of weight, definite fecal changes and a depression of the reproductive system.

Hen B3 presented the most pronounced morbid condition of the 3 cases. Inanition was progressive to the point of a weight loss of 469 g. All symptoms developed progressively worse in the 17 days from the time the first signs of the "leg-weakness" were noted to the time the first injection of ascorbic acid was made. Subcutaneous injections of 100 mg of ascorbic acid in 2 ml of Ringer's solution were made every second day. A total of 1000 mg of the vitamin C in 10 injections were used. Recovery was dramatic. Hen B3 laid the first egg the day following the sixth injection which was on the tenth day subsequent to the first injection. Nineteen eggs were laid by Hen B3 in the following 36 days prior to its death (Table I). Death was due to a hemorrhage from a heart puncture made the preceding day.

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[6] Bell, T. A., Thesis, N.C. State College of Agr. and Eng., 1940.

[7] Parrish, C. F., Maupin, C. J., and Brown, T. T., *Feed Formulas for Poultry*, N.C. Agr. Ext. Ser., Ext. Cir., 1940, No. 245.

[8] Bessey, O. A., and King, C. G., *J. Biol. Chem.*, 1933, **103**, 687.

[9] Guyer, M. F., *Animal Micrology*, 3d ed., 243.

TABLE I.

Hen No.	B3	C1	C2
Hen in confinement	1/7/39	1/7/39	1/7/39
Exper. started	1/14	1/14	1/14
Days on diet prior to ''leg-weakness''	62	67	68
Total eggs 2d laying year prior to ''leg-weakness''	123	163	117
1st day of ''leg-weakness''	3/17	3/22	3/23
No. days ''leg-weakness'' prior to ascorbic acid inj.	17	7	5
Date 1st inj.	4/4	3/29	3/28
Date last inj.	4/20	4/16	4/15
Amt. ascorbic acid inj.	1000 mg	1000 mg	1000 mg
No. inj. weekly	4	4	4
Total No. inj.	10	10	10
Days between 1st inj. and 1st egg	10	6	14
Eggs laid after 1st inj. to disposition	19	36	25
Date of disposition of hen	Died 5/21	Transferred 6/8	Transferred 6/8
Total eggs laid 1st yr	208	254	208
Total eggs 2d laying yr to date of disposition	144	205	157

Hens C1 and C2 received the same treatment as hen B3 and they made the same rapid recovery (Table I).

A fourth case, a S. C. White Leghorn hen, Ex. 25, unrelated to the experiment just described had a case history almost identical to the 3 cases mentioned.

It is the opinion of the authors that certain hens, under the demands of heavy egg production and fed a diet deficient in vitamin C, develop avitaminosis C resulting in "leg-weakness", inanition and a depression of egg production.

Experiments should be conducted to ascertain whether the effect of the injections is due to the ascorbic acid alone, to the salts in Ringer's solutions or to both. Minimum vitamin C requirements of high egg-producing hens for the prevention of "leg-weakness" should be determined. A diet should be formulated incorporating vitamin C-containing foodstuffs fulfilling the necessary vitamin C requirements for the prevention of "leg-weakness", inanition and the depression of the reproductive system in hens bred for high egg production.

13362

Effect of Gradually Increased Concentrations of Sulfathiazole on the Gonococcus *in vitro*.*

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Recent studies[1, 2] have shown that many strains of the gonococcus readily acquire fastness to sulfanilamide and to sulfapyridine when cultivated in a medium to which gradually increased amounts of the drugs are added. It was observed, furthermore, that the strains tested retained this drug-fastness for at least 4 months after subsequent growth on a medium free from the compound. In addition, Bang and Bang[3] observed that 2 of these sulfanilamide-fast strains which they studied had retained their resistance for at least one year.

The present study employing sulfathiazole was undertaken after this drug was proved to be more effective for the treatment of gonococcal infection than other sulfonamide compounds.

Materials and Methods. Ten strains of the gonococcus, recovered from various types of gonococcal infection, were studied. Six strains had been isolated from 1 to 3 years prior to the experiment, and 4 others had been under artificial cultivation for only 3 months. Seven of the strains were isolated from the genito-urinary tract of males, 1 from the cervix, 1 from the vagina of a child, and 1 from the conjunctiva.

The technic used to determine sulfathiazole-fastness was similar to that followed in previous studies on the development of sulfanilamide- and sulfapyridine-fast strains of the gonococcus.[1, 2] The basic culture medium was a pork infusion broth containing 0.5%

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* Studies in coöperation with the Division of Venereal Diseases of the United States Public Health Service.

† On leave of absence from the School of Medicine and Dentistry of the University of Rochester as a Fellow of the Division of Medical Sciences of The Rockefeller Foundation.

‡ On leave of absence from the Oregon State Board of Health, Division of Venereal Disease Control.

[1] Boak, R. A., Charles, R. L., and Carpenter, C. M., Pub. No. 11, American Society for the Advancement of Science, Lancaster, Pa., The Science Press, 1939, p. 118.

[2] Westphal, L., Charles, R. L., and Carpenter, C. M., *Ven. Dis. Inf.*, 1940, **21**, 183.

[3] Bang, F. B., and Bang, B., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 527.

of potassium nitrate, 0.5% of potassium acid phosphate, and 5% of defibrinated rabbit blood. Sulfathiazole was added to yield final concentrations of 0.0005%, 0.001%, 0.002%, 0.003%, 0.004%, and 0.005%.

Each strain of the gonococcus was cultivated at 35°C for 24 hours on a chocolate blood agar slant. The growth was suspended in 1 ml of the infusion broth without rabbit blood, and the suspension immediately transferred to 3 ml of the infusion-blood broth. After 48 hours of incubation, inocula of 0.1 ml were transferred to a tube of the same medium, which served as a control, and to a series of tubes containing 0.0005%, 0.001%, 0.002%, 0.003%, and 0.005% concentrations of sulfathiazole. Tests for viability were made 24 hours after inoculation by means of subcultures on chocolate-blood agar plates. When growth became established at a given concentration of the drug, the strain was introduced into the medium in which the concentration was 0.0005% to 0.003% higher. At the same time, in order to safeguard the maintenance of the culture, it was also inoculated into a tube of broth containing an equivalent amount of drug in which growth had been established. Thus, transfers to the same and to higher concentrations were made every 48 hours, while subcultures for viability were made at the same intervals, but on alternate days. Throughout the study, control cultures were maintained in the infusion-blood broth. The study was in progress for approximately 3 months.

To ascertain if the strains selected for the study would acquire tolerance for sulfanilamide, 8 of them were grown in increasing concentrations of that compound. After it was demonstrated that all had become sulfanilamide-fast, they were inoculated into a blood broth containing 0.005%, 0.01%, and 0.025% concentrations of sulfathiazole to determine if simultaneously they had acquired tolerance for sulfathiazole.

Results. Ten strains of the gonococcus cultivated in gradually increasing concentrations of sulfathiazole grew moderately well in 0.0005%, 0.001%, and 0.002%. At levels of 0.003% and 0.005%, growth did not become established, although on some attempts the strain remained viable for 2 or 4, or in one instance 10, days at these higher concentrations. The cultures were grown in a 0.002% concentration for from 30 to 74 days before being transferred to the higher concentrations. This is in marked contrast to the 8 strains that were grown simultaneously in gradually increasing concentrations of sulfanilamide. Within 14 days, 2 grew in a 0.05% concentration of the drug, 3 in a 0.035% concentration, 2 in a 0.03% concentration, and 1 in a 0.02% concentration. In every instance growth was luxuriant. When the 8 sulfanilamide-tolerant strains were introduced into broth containing sulfathiazole in concentrations of 0.005%, 0.01%, and 0.025%, no growth occurred.

Discussion. The results of this study are striking in that the strains of the gonococcus studied acquired only slight, if any, tolerance for sulfathiazole, although resistance to sulfanilamide was readily established. Even though attempts were made repeatedly over a period of 3 months to establish growth in a medium containing sulfathiazole, success was attained at only the lowest concentrations, *i. e.,* 0.0005 to 0.002%. It is apparent that sulfathiazole exerts a more marked bacteriostatic and bactericidal action on the gonococcus than either sulfapyridine or sulfanilamide. Lawrence[4] has reported that a 0.001% concentration of the drug inhibits the development of the gonococcus in 5 hours.

These *in vitro* studies are in accord with clinical observations of the superior chemotherapeutic action of sulfathiazole in gonococcal infection. The failure of the gonococcus to develop resistance to sulfathiazole *in vitro* suggests that sulfathiazole-fast strains of the organism are less likely to become established in the population at large than are sulfanilamide- or sulfapyridine-fast strains.

Summary. Ten strains of *Neisseria gonorrhoeae* developed little, if any, tolerance for sulfathiazole when grown for approximately 3 months in a blood broth containing concentrations of the drug varying from 0.0005% to 0.005%.

13363 P

Changes in Blood Urea Following Hypothalamic Lesions.

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From the Medical Division, Montefiore Hospital, New York City, and the Department of Experimental Neurology, D. J. McCarthy Foundation, Temple University School of Medicine, Philadelphia, Pa.*

The following observations are reported as part of a study of the influence of diencephalic lesions upon the nitrogenous constituents of the blood.

Methods and Material. Eight cats received lesions in the pre-

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[4] Lawrence, C. A., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 92.

* Aid from the Dudley D. Sicher Fund is acknowledged with thanks.

optic region and the anterior hypothalamic area; 11 cats, lesions in the posterior hypothalamic, tuberal or subthalamic regions; and 11 were controls. All animals received daily about 150 g kidney or beef and 75 cc milk. Blood samples obtained by heart puncture without anesthesia were examined for albumin, globulin, urea, N.P.N., creatinine, cholesterol, chlorides, and sugar; also estimations were made of the hemoglobin, erythrocytes, leucocytes, platelets, and differential counts; in a few instances the blood volume was measured but with results too inconclusive to merit reporting.

Under intraperitoneal nembutal anesthesia, supplemented when necessary by light ether anesthesia, bilateral lesions, symmetrical as nearly as possible, were placed by means of a modified Horsley-Clarke apparatus.[1] Blood studies were made preoperatively and continued from the first postoperative day, in some cases from the second or later days. A majority of the animals reacted badly to the operation, refusing food and drink and losing weight sharply. After 5-7 days they began to improve and regain weight. Withdrawing blood at too frequent intervals produced a marked anemia, this disappeared when the bleedings were discontinued.

Results. A rise in blood urea was noted in 7 of 8 cats with lesions of the anterior hypothalamic and the preoptic area (Table I). The exception, cat No. 16, proved to have widely asymmetric lesions. The rise in urea was paralleled by a rise in N.P.N., the latter representing exactly the increase of urea N. All other substances tested in the blood remained at *normal levels.* Cat No. 13 was of particular interest. Daily blood urea figures, normal for several weeks before operation, became abnormally increased during the 31-89th post-operative days. The animal appeared to be in excellent health although it grew very fat (confirmed at autopsy). The urine was

TABLE I.
Blood Urea of Cats with Anterior Hypothalamic and Preoptic Lesions.

Cat	Pre-op. urea	Post-op. urea	Post-op. days survival
7	23.0	62 (4); 46 (11); 34.4 (16); 22.5 (23)	27
9	25.8	15.6 (1); 44 (4); 58.2 (7)	8
10	25.6	11.5 (1); 100 (3)	5
11	22.1	20.4 (1); 28.0 (5); 49.3 (6); 28.4 (7); 24.1 (8)	18
13	22.7	Normal (1-9); 46.6-55.0 (31-87); 29.0 (108)	113
15	19.0	11.6 (2); 18.1 (9); 38.3 (14); 62.0 (15)	15
16	28.3	14.3 (1); 11.8 (5)	6
19	19.8	34.0 (2); 72.0 (3)	3.5

Figures in () designate number of post-operative days.

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[1] Spiegel, E. A., and Miller, H. R., *J. Lab. and Clin. Med.*, 1941, **26**, 1657.

TABLE II.
Blood Urea of Cats with Lesions in

Cat	Pre-op. urea	Post-op. urea	Post-op. days survival
		(a) Posterior Hypothalamus.	
5	25.4	27.2 (2); 27.2 (86)	92
12	20.4	25.4 (2); 29.6 (5)	6
15A	22.0	15.0 (2); 19.0 (39)	59
17	14.1	15.0 (2)	2
20	23.0	22.7 (2); 19.6 (19)	19
		(b) Subthalamus.	
2	40.1	20.8 (4); 24.0 (17)	32
5A	28.8	26.4 (3); 27.2 (53)	64
12A	20.4	24.0 (2); 23.4 (32)	41
		(c) Tuberal Region.	
1	24.8	20.1 (2); 34.4 (18)	24
4	26.1	28.0 (9); 38.2 (28)	30
21	25.0	22.5 (2); 22.0 (4)	4

Figures in () designate number of post-operative days.

normal. Blood urea fell to normal on the 108th day. On the 113th day the cat was accidentally killed during cardiac puncture. Macroscopically the kidneys were unusually pale and fatty in appearance with fine irregular surface and marked lobulations. Microscopic studies of the kidneys and other organs are in progress. Marked obesity developed also in one cat (with tuberal lesion) with normal urea level observed for 30 days after operation, indicating that development of obesity and rise of urea may be independent phenomena.

No rise in urea or in other blood elements thus far studied followed the lesions in the posterior hypothalamus, in the subthalamus, in the tuberal region, or in "combined" diencephalic lesions in the posterior hypothalamic and tuberal region (cats Nos. 5, 12, 15a). In 11 control experiments, *i. e.*, after starvation up to 10 days or anesthesia, anemia following frequent bleedings (hemoglobin 40%), unilateral lesions in the anterior or in other hypothalamic areas, or in the presence of purulent infection, the urea level remained normal. There was one instance of "spontaneous" increase in blood urea; the urea was 200 and 150 mg per 100 cc on 2 different days. This animal appeared to be fairly old and sick; it was not operated on. Autopsy disclosed a left shriveled, atrophic kidney, the right markedly hypertrophied.

Summary. 1. Lesions of the anterior hypothalamic area and preoptic region were associated with a definite and apparently isolated rise in blood urea, no change being noted in the level of the other blood substances tested. 2. In 3 animals surviving beyond 18 days the elevated blood urea returned to a normal level (Table I). 3. With one exception the kidneys of the operated animals were macroscopically normal. 4. No apparent interdependence was discerned between the level of blood urea and the state of severe anemia or between the former and body temperature or the development of obesity. The disturbance in the level of blood urea presumably is not associated with any discernible metabolic disorder.

The rise in blood urea may be due to an impairment of the ability of the kidney to clear urea at normal blood level; an increased blood urea may be a prerequisite for the adequate elimination of this substance when lesions are made in the anterior hypothalamic and preoptic regions.

13364 P

Precipitation of Active Influenza A Virus from Extra-Embryonic Fluids by Protamine.

Leslie A. Chambers* and Werner Henle. (Introduced by D. W. Bronk.)

From the Department of Pediatrics, University of Pennsylvania, and the Children's Hospital, Philadelphia, Pa.

The allantoic and amniotic fluids of infected chick embryos have been found to contain large amounts of both active influenza virus and the complement fixation antigen[1, 2] in spite of their low total protein content. These extra-embryonic fluids, either fresh or lyophilized, constitute a very adequate antigen for the diagnosis of influenza by complement fixation test, and the suggestion has been made by us that they should be superior to the usual tissue emulsions as vaccines because of the relatively small amount of cellular debris.

We have carried out a series of experiments to determine the degree of protection afforded to mice by intraperitoneal vaccination with infected extra-embryonic fluids. Five-week-old Swiss mice were inoculated once with 0.05 cc of pooled dialyzed fluids. The fluids used were infectious for mice in dilutions of 10^{-8} to 10^{-10} and contained about 0.20 mg of N per ml, dialysis having removed the large amount of uric acid nitrogen originally present.

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* Fellow of the Nemours Foundation.

[1] Henle, W., and Chambers, L. A., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 713.

[2] Nigg, C., Crowley, J. H., and Wilson, D. E., *J. Immunol.*, 1941, **42**, 51.

Approximately 3 weeks subsequent to vaccination the mice were inoculated intranasally with 0.05 cc amounts of serial decimal dilutions of egg fluid infected with the homologous strain. In a series of experiments the vaccination resulted in protection of the mice against as much as 10^8 lethal doses of virus when test inocula of adequate strength were used.

While dialysis eliminates the uric acid, non-dialyzable substances other than virus remain in the fluids in relatively large amounts. The presence of egg proteins can be shown by precipitation with rabbit sera against ovalbumin or whole egg white.

We have attempted to separate the virus from these adventitious substances by various adsorptive and precipitative procedures which will be discussed elsewhere. One practicable procedure of immediate interest consists in precipitation of the active substance by the addition of protamine.

When about 1/5 of a volume of an aqueous solution (0.5 mg/cc) of a basic protein, such as spermine, is added to the infected egg fluid harvested from living embryos, a precipitate appears immediately. Precipitation can be effected either before or after dialysis of the fluid, but occurs only in the pH range 7.0-9.0. Fluids from normal eggs yield no precipitate or only a very slight amount.

The precipitate is sedimentable in the angle centrifuge at 5000 rpm. Since it is readily resuspended several washings may be carried out. Upon resuspension to the volume of the original fluid the precipitated material shows approximately the same infectivity as the original fluid although analysis shows that the suspension contains only about 0.4% of the original nitrogen. Some preparations have produced infection in mice in doses of 10^{-16} g (dry weight).

Immediately after precipitation the protamine-virus complex was resuspended to the original fluid volume and used as a vaccine for mice with the results indicated in Table I. Mouse protection appeared to be equal to that afforded by the whole extra-embryonic fluid despite the fact that only a small fraction of the total egg fluid protein was inoculated. Quantitative comparisons of the 2 vaccines are in progress.

The active agent in this form is distinctly different from preparations sedimented from infected lungs.[3] It is relatively simple in structure consisting largely of ribonucleoprotein. Analysis of the

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[3] Chambers, L. A., and Henle, W., *Am. J. Path.*, 1941, **17**, 442.

TABLE I.

Protamine Vaccination. The effect of intranasal administration of 0.05 cc of the test virus solution to the vaccinated and unvaccinated groups of mice is indicated. D indicates death, the subscript showing the days of survival after infection. 4, 3, 2, and 1 indicate different degrees of lung consolidation on autopsy after 10 days.

Vaccinated with F-12-33 (6/3/41)	I.N. M.I.D.*	Tested with F-12-33 (6/23/41)	Results: Mouse No.									
			1	2	3	4	5	6	7	8	9	10
Original	10^{10}	undil.	3	2	1	±	±	0	0	0	0	0
		10^{-1}	±	±	0	0	0	0	0	0	0	0
Protamine Sediment	10^{9}	undil.	1	1	±	±	0	0	0	0	0	0
		10^{-1}	0	0	0	0	0	0	0	0	0	0
Not vaccinated		10^{-4}	D_6	D_6	D_7	D_9	D_{10}	D_{10}	D_{10}	4	3	3
		10^{-5}	D_4	D_7	D_9	D_9	D_9	D_{10}	3	3	2	2
		10^{-6}	4	4	4	3	3	3	3	2	1	1
		10^{-7}	3	3	3	3	2	2	1	1	0	0
		10^{-8}	2	1	1	0	0	0	0	0	0	0

*Intranasal maximal infectious dilution.

complex is being carried out. The dry weight figure of 10^{-16} g as an infectious dose limits the molecular weight of the complex to the order of 10^8 or less and a series of electron-micrographs,[†] to be described in a later publication, shows that the resuspended complex contains no particles of diameter greater than 15 mμ, but a considerable amount of aggregation was apparent.

In spite of the relatively high degree of homogeneity obtained by the procedure described, the material still contains complement fixation antigen.

It appears that protamine precipitation of the active agent of influenza from infected egg fluids constitutes a step toward isolation, and identification of the virus. The usefulness of the complex in either an active or inactivated form as a vaccine is indicated, and its efficacy in the protection of human subjects will be investigated.

[†] Taken by Dr. T. F. Anderson in the laboratories of the R. C. A. Manufacturing Company.

13365 P

A New Serologically Active Phospholipid from Beef Heart.

MARY C. PANGBORN. (Introduced by Augustus B. Wadsworth.)

From the Division of Laboratories and Research, New York State Department of Health, Albany.

Alcoholic extracts of beef heart have long been used as antigens in the complement-fixation test for syphilis, but the substances responsible for the serologic activity of such extracts have not been identified. The isolation of a new phospholipid is here reported and evidence is presented that the new substance is the active component of the antigenic extracts. Details of its preparation and chemical properties will be published elsewhere.

The substance was prepared by precipitating alcoholic extracts of beef heart with cadmium chloride, removing lecithin from the precipitate by a recently described method[1] and fractionating the lecithin-free cadmium compounds by precipitation from ether with alcohol and acetone. The purest preparation of the cadmium-free phosphatide contained 4.18% of phosphorus and no nitrogen. It was subjected to repeated purification in the form of the cadmium salt without essential change in chemical or serologic properties and is therefore considered to be a single substance.

An abbreviated complement-fixation technic[2, 3] based on the quantitative methods of Wadsworth, Maltaner, and Maltaner[4] was employed in testing the serologic activity. High-titered sera from syphilitic patients, diluted in pooled, filtered, normal sera, were used. Control tests with pooled sera from nonsyphilitic persons were negative.

In testing the complement-fixing activity of lipid fractions that have been freed of lecithin, it is necessary to add both lecithin and cholesterol. In the absence of lecithin, such fractions are markedly anticomplementary; in the absence of cholesterol, the purified fractions have not been found to fix complement. The amount of added lecithin was so adjusted that a 1:10 saline dilution of the antigen was not anticomplementary. Alcoholic solutions were made up to contain 0.03% of the test substance, 0.05% of lecithin, and 0.4% of cholesterol, and were diluted with 0.85% sodium chloride by the method used for the routine antigen.[2]

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[1] Pangborn, M. C., *J. Biol. Chem.*, 1941, **137**, 545.

[2] Wadsworth, A. B., *Standard Methods of the Division of Laboratories and Research of the New York State Department of Health*, 2d ed., Baltimore, The Williams and Wilkins Company, 1939, pp. 213-267.

[3] Kent, J. F., *J. Immunol.*, 1940, **39**, 307.

[4] Wadsworth, A., Maltaner, F., and Maltaner, E., *J. Immunol.*, 1938, **35**, 105, 217.

The lecithin used was purified by the petroleum ether-alcohol method.[1] Lecithin so purified is not anticomplementary and fixes no complement in the presence of syphilitic or normal sera, either alone or when mixed with cholesterol. Lecithin from eggs and from beef heart gave identical results.

Comparisons of antigenic activity were based upon the linear relation of syphilitic serum to complement. A series of serum dilutions was titrated, using several antigen dilutions with each quantity of complement; the range of antigen dilutions was so chosen as to ensure a maximum reaction with each amount of serum.[3] From the observed percentages of hemolysis given by each serum quantity with its optimum dilution of antigen, the number of units* of complement required for 50% hemolysis was determined,[2, 3] and the values so obtained were plotted as ordinates against quantities

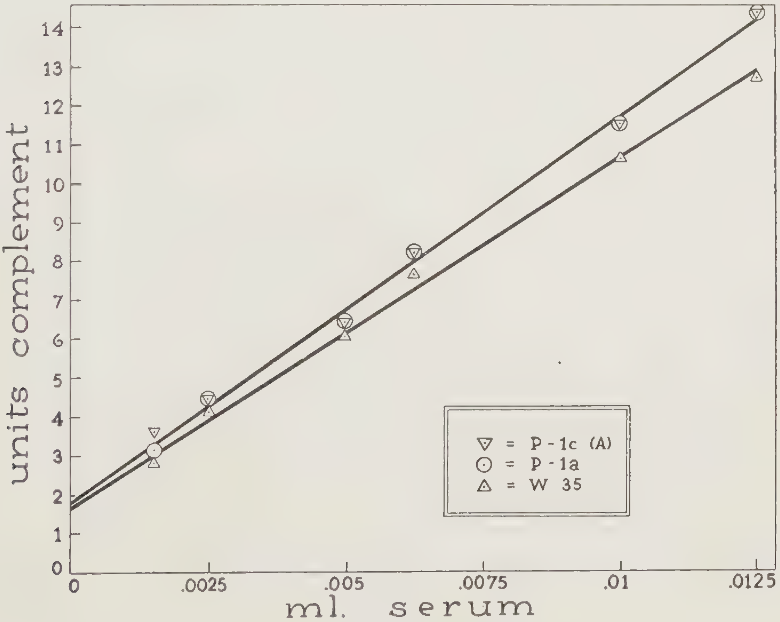

FIG. 1.
Titration of syphilitic serum with purified phospholipid.

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* One unit of complement is the amount required for 50% hemolysis of 0.1 ml of a 5% suspension of sheep erythrocytes sensitized with an equal volume of optimally diluted amboceptor.[2]

of serum as abscissae. Equally active antigens simultaneously titrated in this way with the same serum and the same pool of complement should give identical serum-complement curves; any significant difference in slope indicates a difference in the complement-fixing activity of the antigens under the conditions of the experiment.

The figure presents a titration in which 2 samples of the purified phosphatide are compared with the standard antigen W35, which was prepared for routine diagnostic use.[2] Of the 2 preparations, P-1c(A) was the more highly purified; it was obtained from P-1a by repeating the purification of the cadmium salt. The serologic activity was evidently not altered by the final purification, since the 2 preparations gave identical titration curves.

The figure shows that the activity of the purified substance is closely comparable to that of the routine antigen, and it may be concluded that the new phosphatide is responsible for the complement-fixing activity of beef heart extracts with syphilitic sera. Chemical and serologic studies of the substance are being continued.

13366 P

Effect of Pseudopregnancy on Mammary Carcinoma Incidence in Mice of the A Stock.

L. W. LAW.* (Introduced by C. C. Little.)

From the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

The inbred A stock of mice has a high incidence of spontaneous mammary carcinoma when there occurs a hormonal stimulation of the mammary tissue resulting from breeding. Females kept as virgins, however, have a relatively low incidence of breast cancer. The incidence of breeding females of a selected subline (F38-F58 generations) was found to be 96.3% and for virgin females, 4.9%.[1, 2]

Fifty pedigreed females, 2 months of age, of a selected subline (F38-F58 generations) obtained from Dr. J. J. Bittner were placed 4 in a cage with a vasectomized A stock male. Oestrus smears were made on mice after discovery of vaginal plugs. All experimental mice were known to be in a state of pseudopregnancy at least once.

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* Finney-Howell Foundation Fellow.

[1] Bittner, J. J., *Pub. Health Rep.*, 1939, **54**, 1642.

[2] Bittner, J. J., *Cancer Research*, 1941, **1**, 113.

The average length of the pseudopregnant condition was found to be 9.9 days with a range of from 5 to 12 days. This is similar to the length of the pseudopregnant state observed in mice by Turner and Gomez.[3] There occurred in these animals a distinct hyperplasia of the duct system and some growth of the gland lobules.

Of 43 mice which were living after the appearance of the first tumor, 11 or 25.6% developed mammary carcinoma. The first tumor appeared at 245 days of age, the last at 622 days. The average age of appearance was 14.6 months. The average age of life in months of cancerous mice was 15.7 which is approximately midway between the cancer age of virgin and breeding mice of this selected subline. The average age of non-cancerous mice was 17.5 months, which likewise is between the non-cancer age of breeding and virgin females of this strain.

All tumors were of the adenocarcinoma type. Nine of these were adenocarcinoma simplex and 2 papillary cyst adenocarcinomas.

Any attempt at this time to explain the factors operating in increasing the incidence of mammary carcinoma in mice subjected to pseudopregnancy would be premature. Certain conclusions, however, can be drawn. Production of young *per se* is not a necessary antecedent for the development of breast cancer in the A stock. Given a certain amount of mammary gland growth, produced by the pseudopregnant condition (probably comparable to the growth of the mammary gland during the first half of pregnancy) a significant increase in the breast cancer incidence occurs. Other factors accompanying pregnancy must be acting to produce the physiological state of the mammary tissue cells necessary for complete cancerous transformation within the stock.

Summary. A definite increase in the mammary carcinoma incidence occurred in females of the A stock following induced pseudopregnancy by mating with vasectomized males. Eleven or 25.6% of 43 experimental mice developed breast adenocarcinomas at an average age of 14.6 months.

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[3] Turner, C. W., and Gomez, E. T., *Missouri Agric. Exp. Sta. Research Bull.*, 1933, 182.

13367

Metabolic Studies in Patients with Gastrointestinal Cancer. III. The Hepatic Concentrations of Vitamin A.*

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In a previous communication[1] the fact was demonstrated that 85% of patients with cancer of the gastrointestinal tract had abnormally low plasma levels of vitamin A. In more than half of the patients this abnormality could not be explained by a deficient ingestion or malabsorption of the vitamin. Since there is considerable evidence to show that the liver is intimately concerned with the storage, distribution, and formation of vitamin A,[2] it was thought that the low plasma levels of the vitamin probably reflected, in those patients, hepatic insufficiency. Subsequently, it was demonstrated that a considerable degree of hepatic dysfunction did exist in patients with gastrointestinal cancer,[3] but whether that dysfunction had resulted in a decreased capacity of the liver to store vitamin A or to distribute the vitamin properly, still remained unknown.

Recently an opportunity was presented to measure in patients with gastrointestinal cancer the vitamin A content of samples of liver removed at the time of laparotomy, and thus to ascertain whether or not the low levels of vitamin A in the plasma of the patients studied were due to decreased hepatic stores or to some other cause.

Clinical Material. The clinical material studied comprised 2 groups of individuals. The first consisted of 21 adults who were used as controls, 14 males and 7 females. Of the 21 individuals, death was due to skull fracture in 16, coronary occlusion in 3, gunshot in 1, and stab wound in 1. All these individuals were autopsied and sections of liver were obtained within 24 hours after death. Other than the immediate cause of death no gross pathological changes were noted.

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* The authors gratefully acknowledge the assistance given by Standard Brands, Inc., the Jane Coffin Childs Fund and the Dazian Foundation.

† Finney-Howell Fellow.

[1] Abels, J. C., Gorham, A. T., Pack, G. T., and Rhoads, C. P., *J. C. I.*, 1941, **20**, 749.

[2] Moore, T., *Biochem. J.*, 1931, **25**, 275.

[3] Abels, J. C., Rekers, P. E., Binkley, G. E., Pack, G. T., and Rhoads, C. P., in press.

The second group was composed of 14 patients, 13 with gastric cancer and 1 with cancer of the colon. Ten were males and 4 females. Of the 14 patients, only 3 had been on grossly deficient diets before admission, and none had received preoperatively any vitamin A or other dietary supplement.

Methods. The method used for the measurement of plasma vitamin A is that described in the previous communication.[1]

To measure the vitamin A content in the liver, samples of from 100 mg to 300 mg were obtained either at the time of laparotomy or at autopsy. All these samples were taken from the anterior margin of the right lobe. It is recognized that the distribution of the vitamin is not entirely uniform throughout the liver, but it was not feasible or permissible to obtain larger biopsy specimens for study.

As soon as each sample of liver tissue was obtained, it was placed in a tared weighing bottle in order to prevent loss of its weight by evaporation of water. After the weight of the tissue was calculated, the entire sample was homogenized in water and the resulting suspension diluted to 10 ml. Of this liver brei, from 1 to 4 ml were extracted with 10 ml of 95% alcohol and 10 ml of light petrol ether in a 50 ml centrifuge tube. Two ml portions of the petrol ether layer were removed for the determination of vitamin A by the same technic as that used for the determination in plasma. Hepatic concentrations of vitamin A are expressed in U.S.P. units per gram of wet weight.

The content of total lipids in the samples of liver brei was determined by the method of Van Slyke.[4]

Results. A. *Concentration of Vitamin A in Livers of Individuals Used as Controls.* It has been reported frequently that the vitamin A content per gram of normal human wet liver ranges from 200 to 1500 U.S.P. units.[5, 6] Nevertheless, it was desirable to determine by the technic used in this investigation the range of vitamin A concentrations in the livers of the 21 individuals who were chosen as control subjects. The concentrations of the vitamin in their livers ranged from 186 to 1302 U.S.P. units and averaged 722 U.S.P. units per gram (Table I).

Whereas the vitamin A levels in the plasma of normal males is significantly higher than those of females, no such difference was

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[4] Van Slyke, D. D., Page, I. H., and Kirk, E., *J. Biol. Chem.*, 1933, **102**, 635.

[5] Ralli, E. P., Papper, E., Paley, K., and Bauman, E., *Arch. Int. Med.*, 1941, **68**, 102.

[6] Bessey, O. A., and Wolbach, S. B., *The Vitamins*, Am. Med. Assn., Chicago, 1939, 30.

TABLE I.

Concentrations of Vitamin A in Livers of Individuals Used as Control Subjects.

Subject	Sex	Cause of death	Vitamin A U.S.P. units per g of liver	Microscopic examination of liver
F.S.	M	Coronary thrombosis	988	Normal
E.F.	M	” ”	1302	”
E.R.	F	” ”	1161	”
J.B.	M	Skull fracture	193	”
J.L.	M	” ”	805	”
—	M	” ”	1068	”
R.M.	M	” ”	1202	”
M.Q.	M	” ”	800	”
—	M	” ”	186	”
A.F.	M	” ”	234	”
—	M	” ”	848	”
—	M	” ”	989	”
—	M	” ”	1006	”
—	M	” ”	522	”
—	F	” ”	577	”
—	F	” ”	773	”
—	F	” ”	939	”
—	F	” ”	334	”
B.A.	F	” ”	579	”
P.R.	F	Gunshot	445	”
—	M	Stab wound	227	No section
			Average 722 U.S.P. units per g	

noted for the hepatic content of the vitamin. Of the 21 individuals, 14 were males and had hepatic vitamin A concentrations from 186 to 1302 U.S.P. units, and averaged 739 U.S.P. units per gram. The concentration of the vitamin in the livers of 7 females varied from 334 to 1161 U.S.P. units and the average was 700 U.S.P. units per gram. This range of values is in good agreement with those previously reported.

B. *Concentration of Vitamin A in Livers of Patients with Cancer of the Gastrointestinal Tract.* Since it has been found that 85% of patients with gastrointestinal cancer have plasma levels of vitamin A below the normal range, it was important to determine whether or not there also existed abnormal concentrations of the vitamin in the livers of those patients. Accordingly, 14 patients with cancer of the stomach and colon were studied (Table II). The hepatic concentrations of vitamin A ranged from 99 to 2236 U.S.P. units, and averaged 697 U.S.P. units per gram, or 97% of the normal value. Only 2 livers had concentrations below the control range.

On the other hand, of the 14 patients studied, the plasma levels of vitamin A were obtained in 10, and were found to be below the normal range in all but 1 instance. Eight of the 10 patients had distinctly low plasma levels of vitamin A associated with hepatic

TABLE II.
Concentration of Vitamin A in Liver and Plasma of Patients with Gastrointestinal Cancer.

Patient	Sex	Diet	Vitamin A, U.S.P. units		Liver total lipids, g per ml	Microscopic examination of liver
			Per g of liver	Per 100 ml plasma		
H.G.	M	Adequate	1347	95	6.6	Normal
G.P.	M	"	1072	105		Acute focal necrosis
L.G.	M	Deficient	786			Normal
D.S.	M	Adequate	1017	105		"
A.E.	M	"	2236			"
T.F.	M	"	713	120		"
J.Y.	M	"	196	135	3.3	"
L.S.	M	"	1226	75	7.3	"
J.R.	M	Deficient	99	40		"
E.W.	M	Adequate	242	85	6.2	"
F.R.	F	"	195	7	4.4	"
F.L.	F	"	337	90		"
K.P.	F	"	159	95	5.4	"
Y.B.	F	Deficient	948			"
		Average	697 U.S.P. units per g			

concentrations of the vitamin which were within the control group.

It has been found here and elsewhere[5] that the fatty livers of patients with chronic alcoholism contain considerably less vitamin A than do those of normal individuals. It would appear, therefore, that the low plasma level of the vitamin in patients with fatty livers is not due to the fact that the fat-soluble vitamin A was withheld from the blood. Nevertheless, it was of interest to determine whether or not the livers of patients with gastrointestinal cancer had an abnormally high content of lipids. The liver total lipids, accordingly, were determined in 6 of the 14 patients studied, and were found to range from 3.3 to 7.3 g %—well within the normal limits.[7] This finding thus would eliminate the possibility that the low levels of vitamin A in the plasma of patients with gastrointestinal cancer were due to an abnormally high content of liver fat with consequent hepatic retention of the fat-soluble vitamin A.

Discussion. This study was undertaken to determine whether or not the low levels of vitamin A in the plasma of patients with gastrointestinal cancer were due to an inability of the livers of those patients to store the vitamin. The results presented here indicated that there is no gross defect in the hepatic storage of the vitamin in these patients to explain the corresponding low plasma levels. Therefore, another explanation must be sought, and the possibility has been considered in the previous communication[1] that the livers of patients with gastrointestinal cancer might not be able to distribute the vitamin normally. It was demonstrated that the administration of choline, yeast, or lipocaic could raise the levels of the vitamin in the plasma. Since both yeast and lipocaic are known to contain choline, their activity conceivably might be due to that compound, and it is possible that in these patients there might exist a deficiency of a substance with a choline-like activity. This possibility still has to be investigated.

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[7] Ralli, E. P., Rubin, S. H., and Ringler, S., *J. C. I.*, 1941, **20**, 93.

Conclusions. The low plasma levels of vitamin A of patients with cancer of the gastrointestinal tract probably are not due to a decreased capacity of the livers of those patients to store the vitamin.

13368

Relation of Arterial Pulse-Pressure to Arterio-Venous Oxygen Difference, Especially in Arterial Hypertension.

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Pulse-pressure in the brachial artery is no longer regarded as a rough measure of cardiac output. Nor can we assume that it gives information concerning what is happening in other arteries. Further, arterial distensibility decreases and pulse-pressure increases as internal pressure is raised, although deviations due to this factor are small in the range of human blood pressures.[1] Comparisons involving pulse pressures from different individuals (varying in size of vessels, muscle masses, metabolic rates, etc.) taken under varying conditions of temperature, humidity, etc., would seem, therefore, to have little justification.

In spite of these considerations an attempt was made to correlate certain data in our possession obtained from 47 patients suffering from a variety of diseases. In Fig. 1 the arterio-venous oxygen differences have been plotted against the products of the pulse pressure multiplied by pulse rate (PP × PR) as ascertained on the brachial artery. Venous blood was drawn from the median basilic vein. It will be seen that most of these cases fall within a curved area (AA), the mean of which is roughly represented by a curve (DD) such that, for any point on the curve,

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[1] Hallock, P., and Benson, I. C., *J. Clin. Invest.*, 1937, **16**, 595.

$$(PP \times PR) \times (\text{A-V } O_2 \text{ diff}) = K_1, \text{ a constant} \quad (1)$$

The shaded area E represents the "normal" area.

The oxygen consumption of an arm at rest can be assumed to be constant and equal to the product of the volume of blood entering or leaving the arm per minute (V) and the volume of oxygen lost by each 100 cc of blood while traversing the arm, or,

$$V \times (\text{A-V } O_2 \text{ diff}) = K_2 \quad (2)$$

If equation (1) above correctly expresses a general relationship between these 2 variables it follows that V varies directly as, or is proportional to, PP × PR. The latter can therefore be used as a fair clinical indication of blood flow through the arm.

At present we are concerned only with the relationship between pulse pressure and A-V oxygen difference, and not with the individual diseases in which variations in these factors occur or with the

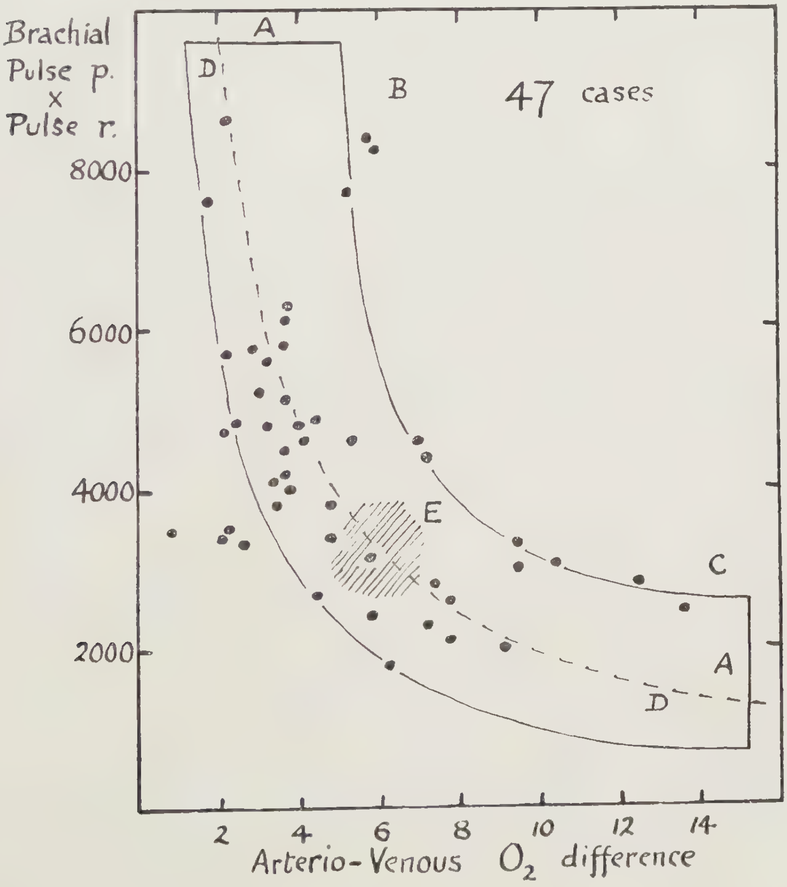

FIG. 1.

reasons thereof. It might, however, be mentioned (a) that those cases falling in the lower portion of the curved area, with circulatory stasis, are all, as might be expected, cases of cardiac disease (valvular disease, calcified pericarditis, emphysema), cancer, certain severe anemias, etc., and (b) that those cases falling in the upper part of the curved area, with a large pulse pressure and a rapid passage of blood through the tissues, are nearly all cases of arterial hypertension and certain other cases of anemia with low diastolic pressures.

In arterial hypertension it has always been assumed that the peripheral vasoconstriction was general throughout the body. Recently, however, Abramson[2] has shown that "in the course of plethysmographic studies on the peripheral vascular responses of hypertensive patients, it was noted that in about 50% of the cases the resting blood flow in the forearm was much greater than in subjects with normal pressure. Such findings differ from those presented previously by Prinzmetal and Wilson,[3] using a similar method, and from those obtained by Pickering,[4] who utilized Stewart's calorimetric procedure. These investigators concluded that the increased vascular resistance in hypertension is generalized throughout the systemic circulation rather than confined to the splanchnic area." Our results, obtained by an entirely different method, showing an increased blood flow through the arm, suggest that, whatever vasoconstriction may occur in the arm, it is relatively less marked than that in the splanchnic area. This interpretation supports Abramson's contentions.

From these results it would seem that, instead of regarding variations in pulse pressure as indications of similar variations in cardiac output, we should rather regard them as indicating the varying use of the arms as a "blood shunt", *i. e.*, that the high pulse-pressure of hypertension and some anemias indicates that a larger (and the low pulse pressure of circulatory failure a lower) proportion of the total blood volume is being shunted through the arms (and possibly all muscle masses). The value of such an arrangement for reciprocally varying muscular and visceral needs is of course obvious.

In 2 cases with increased basal metabolic rate the points fell on the upper right hand edge of the curved area in Fig. 1 as might be expected. As is well known the B.M.R. is also increased in cases where the heart has increased work to do, as in arterial hypertension and in chronic valvular disease. If this higher B.M.R. is due entirely to the increased cardiac work we would expect conditions in the arm to be unaffected and therefore the points on the graph to be scattered along the median curve DD. The fact, however, that the points representing these cases tend to approach the right hand edge (BC) of the curved area indicates, unexpectedly, that the oxygen consumption in the arm is also augmented and that probably the increased metabolism is general.

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[2] Abramson, D. I., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 127.

[3] Prinzmetal, M., and Wilson, C., *J. Clin. Invest.*, 1936, **15**, 63.

[4] Pickering, G. W., *Clin. Sci.*, 1936, **2**, 209.

Conclusions. 1. Since pulse pressure × pulse rate × arteriovenous oxygen difference in the arms of different subjects roughly equals a constant, PP × PR is regarded as a fair index of blood flow entering the arm.

2. Since PP × PR is increased and A-V O_2 diff. is diminished in arterial hypertension and in certain anemias (in both of which total cardiac output is not increased), it follows that a larger proportion than normal of total blood flows through the arms (and possibly all limbs), *i. e.,* in these two conditions peripheral vasoconstriction is less marked in the arms than in the splanchnic area.

3. In hyperthyroidism the points on the graph are moved to the right of the normal curve indicating an increased oxygen use in the arm. The fact that many cases involving increased cardiac work also move to the right show that the increased metabolism in these cases is not due to increased cardiac work alone, but takes place in the arms also and is possibly a general condition.

4. It is suggested that the pulse pressure serves as a rough but valuable indication of the reciprocally varying needs and blood supply of the viscera and muscular masses of the body.

We are indebted to Dr. W. B. Porter, Professor of Medicine, for allowing us to make use of his clinical data.

13369

Acute Effects of Smoking on Respiration and Circulation.

Rolland J. Main.

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During experiments on the diurnal variations of alveolar CO_2 in man, the question arose as to whether smoking could be permitted occasionally, for it is often a hardship for the subject to refrain from smoking when no good evidence is available to contraindicate it. I therefore studied the effects of smoking one cigaret on alveolar CO_2, blood pressure, and heart rate of humans.

The subjects were 6 males in their early twenties. Some of these were smokers, averaging 20 cigarets a day, and the others were relative non-smokers, averaging not more than one or 2 cigarets a day. Complete non-smokers usually have difficulty inhaling smoke and were, therefore, not used. The subjects refrained from smoking, severe exercise and caffeine beverages for at least 3 hours previous to the experiment. Soon after their previous meal of breakfast or lunch, they lay down for from one-half to one hour, since at least 20 minutes is required to attain respiratory equilibrium,[1] until blood pressure and heart rate become constant. They remained supine during the entire experiment. The experiments were terminated before hunger contractions previous to the next meal appeared, to avoid their disturbance of alveolar CO_2.[2] The alveolar CO_2 samples were obtained by the usual Haldane-Priestly technic, using the expiratory sample only. Samples were taken at 10- or 15-minute intervals, except immediately after smoking, when they were taken at intervals of from 1 to 3 minutes, in order to record more accurately the rapidly changing tensions. Blood pressure and heart rate determinations were made at 10-minute intervals except that following smoking they were taken at 5-minute intervals until the values were approaching normal.

To obtain quantitative results, the cigaret was marked with a pencil 23 mm from the mouth end and smoking ceased as soon as the fire reached this point. Although one puff a minute is considered average, I had the subjects take one puff every half minute, inhaling each one deeply, and attempting to inhale at the same depth each time, unless beginning to experience vertigo, when they were allowed to inhale somewhat less deeply. Thus the effects are exaggerated as compared to normal smoking. No attempt was made to determine what variations occurred in depth of inhalation between various individuals. The regular cigarets used were a leading brand containing 2% nicotine, each cigaret weighing 1 g.

As Fig. 1 shows, smoking one cigaret, using the above exaggerated technic, produces a distinct drop in alveolar CO_2 tension and a rise in blood pressure and heart rate. The effects are rather constant in any one individual, but vary considerably between subjects. Oddly, relative non-smokers do not always show a greater effect than smokers, and may show less. This may be due in part to differences

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[1] Soley, M. H., and Shock, N. W., *Am. J. Physiol.*, 1940, **130**, 771.

[2] Main, R. J., *Am. J. Physiol.*, 1937, **119**, 7.

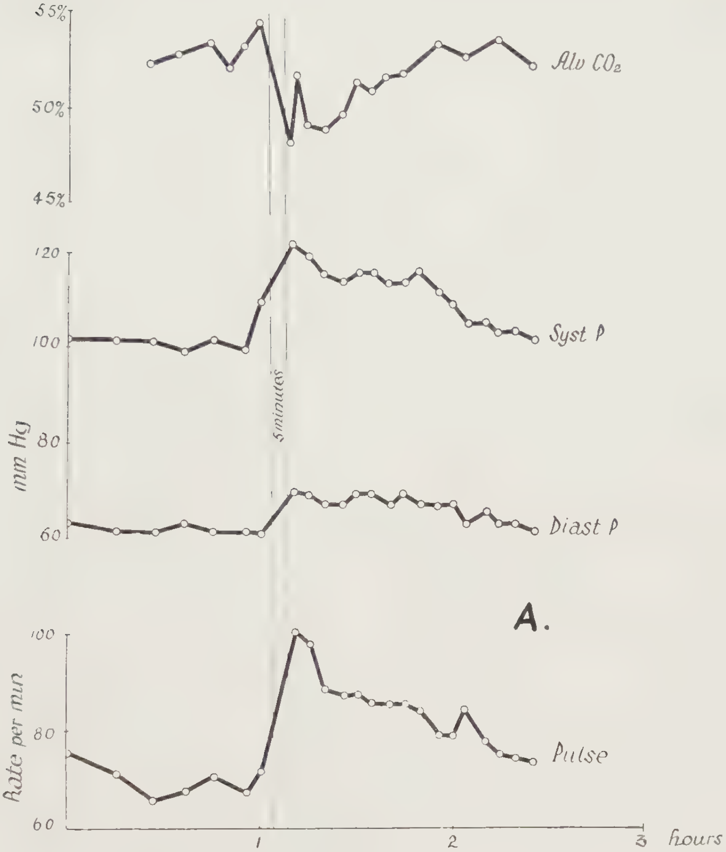

FIG. 1.

A. Response of subject F.L., a smoker, to a regular cigaret. B. Response of subject G.T., a relative non-smoker, to a regular cigaret. C. Response of subject F.L., to a denicotinized followed by a regular cigaret.

The vertical bar represents the duration of smoking.

The time in minutes written vertically on the bar represents the time required to smoke the cigaret.

in amount of smoke inhaled and the time it remains in the lung, or to individual sensitivity. The results indicate that the alveolar CO_2 is depressed for from 10 to 30 minutes following smoking, but that blood pressure and heart rate may not return to normal for from 30 to 60 minutes. These effects are much longer lasting than would be expected from the rapid detoxication of nicotine in rabbits[3] but agree with the results in dogs.[4] These results demonstrate the inadvisability of permitting smoking shortly before or during certain types of physiologic experimentation.

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[3] Weatherby, J. H., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 593.

[4] Biebl, M., Essex, H. E., and Mann, F. C., *Am. J. Physiol.*, 1932, **100**, 167.

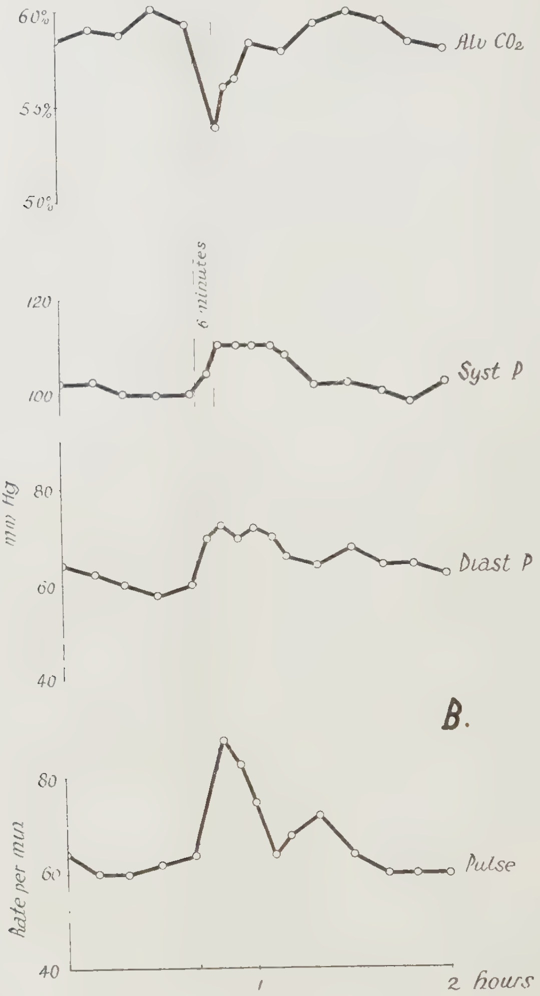

FIG. 1-B.

It then became of interest to determine the nature of the pharmacologic agent responsible for these physiologic disturbances. The substances in cigaret smoke most likely responsible are nicotine, CO, and irritating chemicals. Since nicotine seemed the most logical choice, some completely nicotine-free cigarets of the same brand were made according to the technic to be reported by Weatherby.[5] These cigarets were then tested in the usual manner on 3 of the above subjects. The results (C, Fig. 1) indicate only a negligible effect on respiration and circulation, as compared to a regular cigaret.

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[5] Weatherby, J. H., unpublished data.

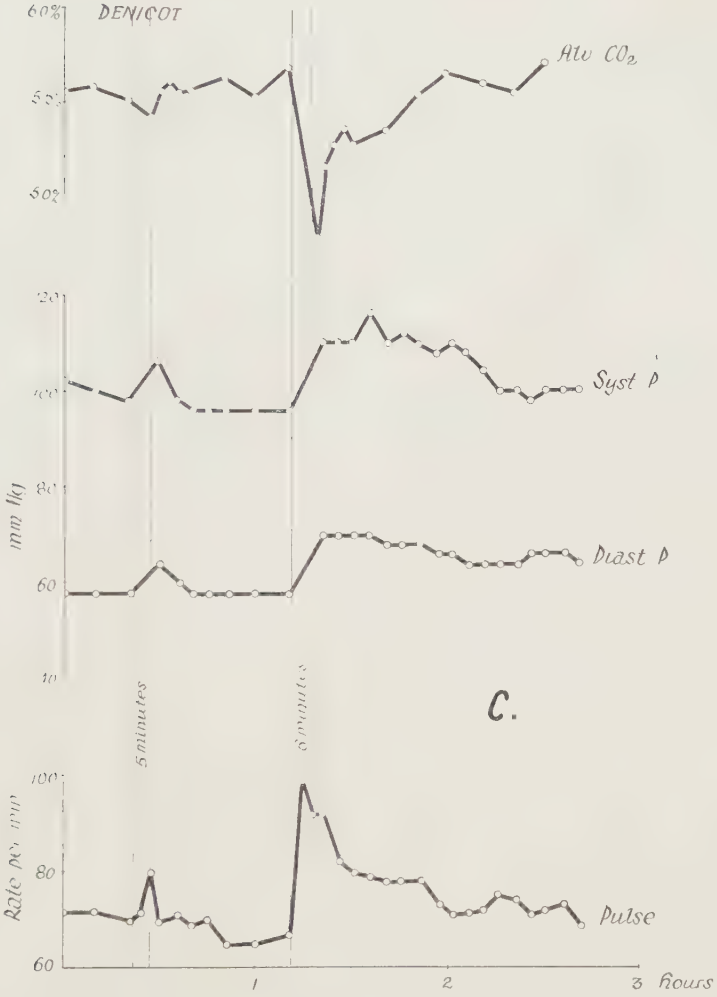

FIG. 1-C.

The effects passed off so quickly that for purposes of comparison, as soon as the subject returned to normal, he would smoke a regular cigaret, as indicated in the figure, (C, Fig. 1). Consequently, I conclude that nicotine is the chief pharmacologic agent in cigarets producing the effects described.

Since it has been suggested[6] that the effect of smoking may be due to nicotine liberating epinephrine from the adrenals, it is of interest to point out that subcutaneously at least, and for an equivalent increase in blood pressure, epinephrine did not produce nearly so marked a tachycardia and had no effect on alveolar CO_2 tension.[7]

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[6] Short, J. J., and Johnson, H. J., *J. Lab. and Clin. Med.*, 1938-39, **24**, 590.

Summary. Smoking one cigaret according to a standardized exaggerated technic, lowers alveolar CO_2 for from 10 to 30 minutes, and increases blood pressure and heart rate for from 30 to 60 minutes in both smokers and relative non-smokers. Nicotine-free cigarets produce very much less effect.

13370 P

Effects of Transplanted Blastemas on Amputated Nerveless Limbs of Urodele Larvae.

Oscar E. Schotté, Elmer G. Butler and Robert T. Hood.
(Introduced by O. C. Glaser.)

From the Departments of Biology of Amherst College and of Princeton University.

Recent histological studies on early stages of Urodele regeneration have revealed that one of the primary consequences of limb amputation is the initiation of dedifferentiation at the amputation level. (Butler,[1, 2] Puckett,[3] and Thornton.[4, 5]) It has been proposed by these authors that dedifferentiated cells contribute to the establishment of the regeneration blastema. The appearance of the blastema coincides with the cessation of dedifferentiation and marks the beginning of the regenerative processes.

Experimental evidence thus far presented indicates that an equilibrium of cellular interaction underlies regenerative activity. In particular, it has been suggested by Butler and Puckett[6] that the blastema is important, not only in the establishment of a regenerate, but also in maintaining a balance between early dedifferentiation and later differentiation. When, by means of either X rays or ultraviolet light, regeneration is prevented, an active blastema never forms. Under such a condition the limb undergoes excessive dedifferentiation. Similar results have recently been obtained by Schotté and

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[7] Main, R. J., Proc. Soc. Exp. Biol. and Med., 1940, **45**, 776.

[1] Butler, E. G., *J. Exp. Zool.*, 1933, **65**, 271.

[2] Butler, E. G., *Anat. Rec.*, 1935, **62**, 295.

[3] Puckett, W. O., *J. Morph.*, 1936, **59**, 173.

[4] Thornton, C. S., *J. Morph.*, 1938a, **62**, 17.

[5] Thornton, C. S., *J. Morph.*, 1938b, **62**, 219.

[6] Butler, E. G., and Puckett, W. O., *J. Exp. Zool.*, 1940, **84**, 223.

Butler[7, 8] in preventing regeneration by denervation of limbs. Since no blastema ever forms on a completely nerveless limb, Butler and Schotté[9] have drawn the conclusion that the excessive dedifferentiation in such limbs is a consequence of the absence of a blastema. It is of particular significance, however, that, if a blastema has been established before the time of radiation or of denervation, although regeneration is prevented, excessive dedifferentiation of structures does not occur. But, up to the present time, no experimental evidence has been available as to whether a transplanted active blastema can exert an influence on dedifferentiation.

The purpose of the present investigation, therefore, has been to transplant blastemas of various ages to nerveless limbs, which ordinarily would undergo extreme dedifferentiation, as demonstrated by Schotté and Butler.[7, 8] A limb of a host larva was denervated by resection of the brachial plexus (left limb, larva C, Fig. 1); soon after denervation the limb was amputated, either through the metacarpals or the forearm region. To the cut surface of amputation was transplanted a regeneration blastema, secured from a previously amputated normal limb (right limb, larva A, Fig. 1).

1. *Transplantation of Blastemas Showing Early Digit Differentiation.* Approximately one hundred transplantations of blastemas showing digit formation have been carried out successfully. The effect of such transplanted blastemas consists essentially in retardation of the rate of limb regression, but not in the cessation of dedifferentiation. The blastema itself was affected by the dedifferentiation of the host limb and eventually was resorbed.

From these experiments it can be concluded that relatively old blastemas, which possess an appreciable amount of differentiation, do not exert an effective regulatory action on dedifferentiation of a nerveless host limb.

2. *Transplantation of Young Blastemas Showing no Evidence of Differentiation.* Results differing markedly from the above were obtained with young blastema transplants. In those cases in which a young transplanted blastema remained squarely in place on the amputation surface of the host limb, little or no regression of limb structures took place. Several cases have been followed for as long as two months, with no appreciable reduction in limb size and no indication of dedifferentiation of skeletal structures (left limb, larva D, Fig. 1). Histological examination of limbs amputated at the

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[7] Schotté, O. E., and Butler, E. G., *Science*, 1941, **93**, 439.

[8] Schotté, O. E., and Butler, E. G., *J. Exp. Zool.*, 1941, **87**, 279.

[9] Butler, E. G., and Schotté, O. E., *J. Exp. Zool.*, 1941, **88**, 307.

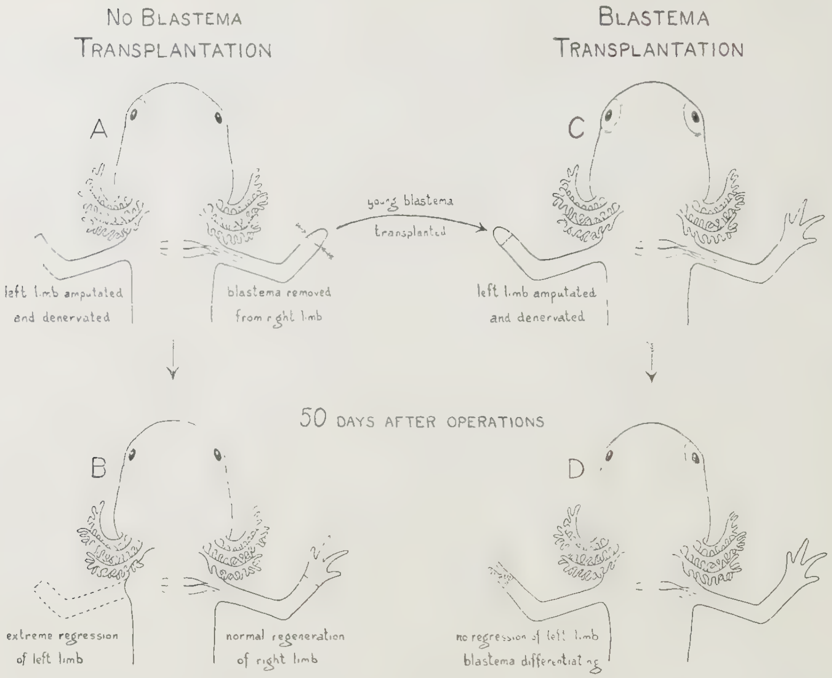
NO BLASTEMA TRANSPLANTATION
BLASTEMA TRANSPLANTATION
A
C
young blastema transplanted
left limb amputated and denervated
blastema removed from right limb
left limb amputated and denervated
50 DAYS AFTER OPERATIONS
B
D
extreme regression of left limb
normal regeneration of right limb
no regression of left limb
blastema differentiating

FIG. 1.

Schematic representation of the described experiments. The larva at the left (A and B) demonstrates the effects of amputation on a nerveless limb (according to Schotté and Butler[8]). The larva at the right (C and D) shows that, if a young blastema is transplanted to a limb which is then kept nerveless for a period of approximately a month and a half, no appreciable reduction occurs. While the transplanted blastema acts as a check to the dedifferentiation of the nerveless limbs, morphogenesis of the blastema, although generally defective, continues.

level of the carpals, to which young blastemas were transplanted, reveals no evidence of dedifferentiation at the end of 2 months. During this period control denervated limbs, amputated at the same level, underwent complete reduction proximally as far as the shoulder (left limb, larva B, Fig. 1).

Conclusions. These results provide new experimental evidence toward a further understanding of the rôle which the blastema plays in regeneration. It has been previously established (Schotté and Butler,[7, 8] Butler and Schotté[9]) that in normal regeneration cessation of dedifferentiation of formed structures of the limb coincides with the appearance of a blastema. It has been demonstrated, furthermore, that when the formation of a blastema is prevented by denervation, dedifferentiation is unchecked and proceeds to extreme ends. In the experiments here reported it is shown that this extreme dedifferentiation in an amputated nerveless limb is prevented by the transplantation of a young blastema.

The presence of a blastema, therefore, is essential for the cessation of dedifferentiation and hence for the establishment of an equilibrium in the cellular interactions which occur during the initial phases of regeneration. This is true, however, only for young undifferentiated blastemas. When a transplanted blastema itself shows unmistakable signs of differentiation, it loses the capacity to control the dedifferentiation of a nerveless amputated limb.

13371 P

Lung Volume and Pulmonary Dynamics in Raynaud's Disease. Effect of Exposure to Cold.

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The occurrence of diffuse pulmonary fibrosis in patients with Raynaud's disease and scleroderma, though rare, has been noted in several instances.[1, 2] The question, therefore, arises as to whether the lung volume and pulmonary dynamics are abnormal in patients with Raynaud's disease and, more particularly, whether exposure to cold affects their pulmonary function. Three patients have been studied; all were females and ranged in age from 15 to 39 years. One of them (Case 3) had cough, dyspnea and X-ray evidence of diffuse pulmonary fibrosis of the type associated with Raynaud's disease.[2] No such findings were present in the other two. All studies were made by the method of Christie,[3] with the patients in the recumbent position. Measurements were made before and again after the patients' legs and at least one hand had been exposed to ice.

Observations. The lung volume and pulmonary dynamics in the 2 patients (Cases 1 and 2) with no clinical or X-ray evidence of pulmonary fibrosis were within normal limits. Case 3, in whom evidences of pulmonary fibrosis were present, exhibited a striking decrease in total pulmonary capacity, vital capacity and complemental air, with a lesser decrease in reserve air; the residual and functional residual airs were within normal limits. The respiratory rate and minute volume were increased.

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[1] Matsui, S., *Mitt. a. d. med. Fakult. d. k. Univ. zu Tokyo*, 1924, **31**, 55.

[2] Linenthal, H., and Talkov, R., *New Eng. J. Med.*, 1941, **224**, 682.

[3] Christie, R. V., *J. Clin. Invest.*, 1932, **11**, 1099.

Exposure to cold caused no change in lung volume or its subdivisions in any of the patients. The respiratory minute volume increased slightly. In addition to the studies here detailed, similar measurements of pulmonary function were made in one case out of doors with the temperature at 40-50°F, with no difference in results.

Discussion. The 2 patients (Cases 1 and 2) with no clinical or X-ray evidence of pulmonary disease showed no physiological evidence of impaired respiratory function. This was in contrast to the findings in the patient with cough, dyspnea and X-ray evidence of fibrosis of the lungs. The complemental air in this patient was greatly decreased, indicating marked impairment of pulmonary distensibility, due apparently to an increased amount of fibrous tissue in the lungs. Attempts to increase the volume of the tidal air in response to the increased demands of exertion would require abnormally strenuous effort and would be only partly successful, thereby leading to dyspnea. Fibrosis also decreases the elasticity of the lungs and further impairs the efficiency of respiration, particularly in regard to expiration; active muscular effort might be required for effective expiration.

The possibility that the increased respiratory activity exhibited by Case 3 might be due to reflexes activated by pathological changes in the pulmonary parenchyma[4] was ruled out by the repeated finding of normal alveolar carbon dioxide concentrations of 5.4 to 5.6%. Dyspnea due to such reflexes is accompanied by a blowing off of carbon dioxide with a resultant decrease in alveolar carbon dioxide concentration. It is probable that the increased respiratory rate and minute volume exhibited by this patient at rest was compensatory to

TABLE I.

Case	Age	Functional residual air cc	Residual air cc	Reserve air cc	Complemental air cc	Vital capacity cc	Total capacity cc	Tidal air cc	Respiratory rate	Respiratory minute volume Liters	Oxygen consumption cc/min	Exposure
1	15	1810	1135	675	2420	3095	4905	260	22	5.7	216	Before
		1695	1100	595	2630	3225	4930	290	22	6.4	207	During
2	24	2310	2030	280	1625	1905	3935	375	18	6.8	180	Before
		2220	1940	280	1585	1865	3805	385	20	7.7	189	During
3	39	1885	1685	200	555	755	2440	345	25	8.6	228	Before
		1910	1705	200	595	795	2500	355	26	9.3	207	During

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[4] Porter, W. T., and Newburgh, L. H., *Am. J. Physiol.*, 1917, **43**, 455.

the lowered efficiency of respiration resulting from impaired distensibility and elasticity. That this compensation was adequate at rest is witnessed by the fact that the arterial blood oxygen saturation was normal, *i. e.*, 94%.

It should be noted that clinical manifestations and physiological measurements indicative of seriously impaired pulmonary function were present months before unequivocal X-ray evidence of fibrosis was detected. Roentgenograms at no time reflected the severity of the pulmonary lesion in this case. This is probably due to the fact that the fibrosis is diffuse, with no large masses or nodules of collagen being formed.

The fact that the lung volume and its subdivisions were not changed when the patients were exposed to cold indicates that the blood vessels of the lungs do not react in a manner similar to those of the hands and feet. This conclusion was strengthened by the observation that exposure to cold did not result in a change in the circulation time through the lungs.

13372

Renin-Like Substance in Blood after Hemorrhage.*

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From the Division of Physiology, University of California Medical School, Berkeley, Calif.

The stimulus adequate to cause the isolated perfused kidney to release renin has been shown by Kohlstaedt and Page[1] to be a fall in the pulse pressure. It seems probable that a similar fall in pulse pressure in the intact animal would affect the kidney similarly. Wakerlin and Chobot[2] were unable, however, to find any change in the renin content of kidneys after their dogs had been subjected to either hemorrhage or splanchnic stimulation. In view of the considerable variation in the renin content of normal kidneys, and of the possibility that the amount of renin liberated is very small in relation to the amount originally present in the kidney, these results

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* This work was aided by the John and Mary Markle Foundation and by the Board of Research of the University of California.

[1] Kohlstaedt and Page, *J. Exp. Med.*, 1940, **72**, 201.

[2] Wakerlin and Chobot, Proc. Soc. Exp. Biol. and Med., 1939, **40**, 331.

cannot be accepted as excluding the possibility that renin is liberated after hemorrhage.

The experiments here reported demonstrate that after hemorrhage the blood has renin-like properties. The rôle of the kidneys, however, remains to be studied.

Methods. The blood was assayed by a modification of a technic originally described by Page[3] for the assay of renin activator. This is based on the observation that whereas either renin or renin activator alone has only slight action on the (rabbit) gut, the two in combination cause a powerful contraction due to the liberation of angiotonin (the gut stimulating agent). Furthermore, gut treated with renin adsorbs enough of it so that even after it is washed an amount remains sufficient to react with added activator and cause contraction.

In the present experiments the guinea pig ileum was used instead of rabbit gut and its behavior appears to be similar. The sensitivity to renin alone, however, seems to be considerably greater than that of rabbit gut.

A section of guinea pig ileum about one inch long is suspended in a bath of warm, flowing, oxygenated Tyrode's solution, its movements being recorded on a kymograph. Introduction of small amounts (0.2 cc) of purified renin[†] is followed by a prolonged slow contraction (Fig. 1A). Angiotonin solutions[†] cause a sharp rapid contraction which soon subsides. Normal blood (renin activator) applied to a previously untreated gut has no effect. If, on the other hand, the gut has previously been treated with renin, normal blood causes a sharp contraction similar in character to that caused by angiotonin (Fig. 1B).

These responses were used as qualitative indicators of the presence of renin and renin activator. No attempt was made to quantitate the assay.

Five dogs were used in the following series of experiments: A preliminary blood sample was taken and heparinized. The dog was then bled from the femoral artery till two-fifths of the blood volume (assumed as 7% of the body weight) had been removed. No medication was used except a local infiltration of the region of the femoral triangle with 1.0% procaine hydrochloride without epinephrine. Signs of cardiovascular distress (rapid, shallow respiration, restlessness, rapid pulse, pallor of gums and tongue) usually appeared

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[3] Page, *Am. J. Physiol.*, 1940, **130**, 29.

[†] The purified renin and angiotonin preparations used in these experiments were generously supplied by Dr. I. H. Page.

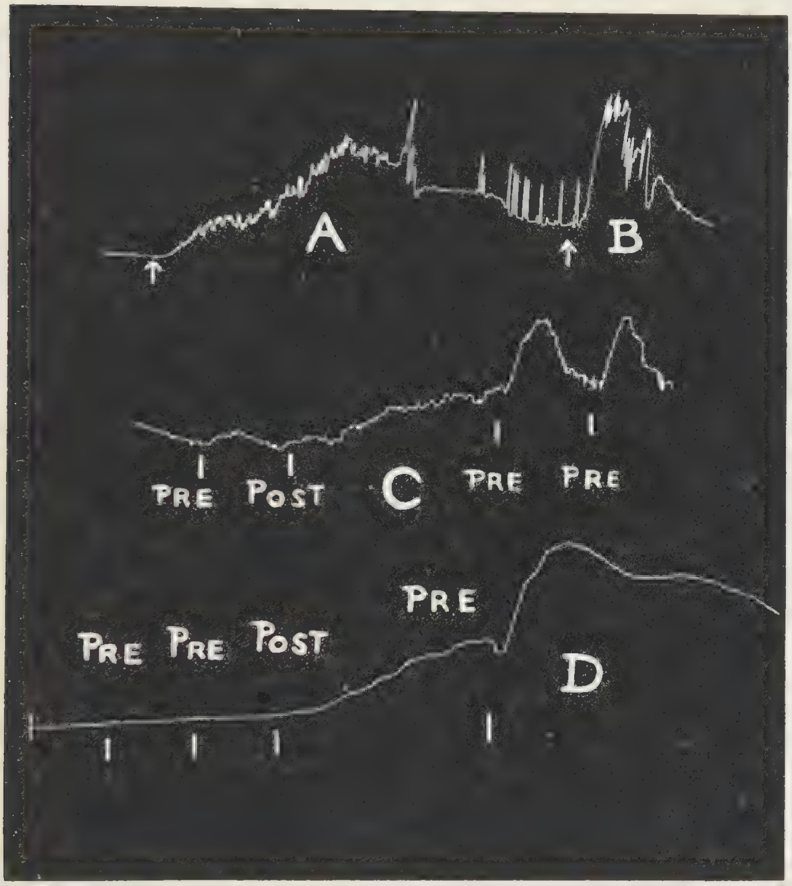

FIG. 1.

A. Response of guinea pig ileum to treatment with .2 cc of a purified renin preparation.†

B. Response to injection of normal blood after preliminary treatment with renin. Note the difference in character of the responses in A and B.

C and D. Responses to normal (pre) and posthaemorrhagic (post) bloods of otherwise untreated guinea pig gut.

before the bleeding was complete. A second blood sample was taken 24 hours after the hemorrhage and heparinized. Both the first and second blood samples were placed on ice immediately after withdrawal. To minimize effects due to difference in storage time the assay of both samples was delayed until 24 hours after the withdrawal of the second sample. The samples were then assayed on the guinea pig ileum prepared as previously described.

Findings. In each of the 5 experiments reported[‡] the 2 samples of blood were tested in varying orders. Table I expresses the findings in 4 of these experiments. Figure 1C shows some of these responses selected for purposes of illustration (see legend).

In the fifth experiment, typical responses were given by the posthemorrhagic blood, but the prehemorrhagic blood was totally inactive on gut "sensitized" by posthemorrhagic blood.

TABLE I.
Tabular Summary of the Typical Result of Exposing the Gut Segment to Pairs of Blood Samples in Varying Order.

1st sample	Effect	Second sample	Effect
normal blood	0	normal blood	0
,, ,,	0	blood after haem.	prolonged slow contr.
Blood after haemorrhage	prolonged slow contraction	normal blood	sharp transitory contraction

Discussion and Conclusions. The observed phenomena are consistent with the concept that the blood of the normal animal, with normal pulse pressure, contains activator but no renin whereas blood taken after hemorrhage when the pulse pressure may be low contains demonstrable amounts of a renin-like substance. Moreover, since the gut always responds slowly to posthemorrhagic blood, it seems likely that this blood contains a diminished amount of activator, since the usual response to a mixture of renin and activator is prompt. Prehemorrhagic blood, which according to this concept contains no renin is incapable of sensitizing the gut to either pre- or posthemorrhagic blood. The results in the single exceptional experiment already cited might be explained by assuming a low renin activator or high renin inhibitor content in the normal blood of the animal in question.

If, as it appears, the renin content of blood increases after hemorrhage, when the effect of such an increase would help restore the blood pressure to normal levels, these findings support the view that the kidney acts as an organ of internal secretion, preserving the homeostasis of the whole circulatory system by a humoral mechanism.

The assay method here described is not specific for renin or renin activator and further experiments to make the identity of the active substances involved more certain are in progress.

[‡] A preliminary group of 6 dogs studied before the technic was adequately standardized gave results which were in all cases compatible with the findings here reported.

13373

Relation of Alcohol Concentration to Intoxication.

HENRY NEWMAN AND MASON ABRAMSON.

*From the Department of Medicine, Stanford University School of Medicine.**

Mellanby[1] made the statement that dogs, after ingestion of a dose of alcohol, manifested a greater degree of drunkenness at a given blood alcohol concentration when this was ascending than they did when the same concentration was again reached on the descent. This observation has been confirmed recently in two publications. Mirsky, *et al.*,[2] found that in both hepatectomized rabbits and normal men, when the blood alcohol concentration was maintained constant over a period the symptoms of drunkenness originally present disappeared, in spite of lack of any change in alcohol concentration. Eggleton[3] demonstrated the same thing in man, and showed, furthermore, that if the alcohol concentration was raised very slowly no symptoms of intoxication appeared even at quite high levels. It was her feeling that the determining factor in the development of intoxication is the rate of increment of alcohol concentration, rather than the actual height achieved. This is probably not fundamentally different from the concept advanced by Mirsky, *et al.*,[2] that the nervous system is able in time to adapt itself to the presence of alcohol in its environment.

Because of the contention by some that alcohol concentration is a reliable index of drunkenness, confirmation of these findings, which if true would completely invalidate such a contention, is of the utmost importance, both from the scientific and the medicolegal aspects, and it was for this reason that the present investigation was instituted.

Two human subjects, both males, were employed. Both could be characterized as occasional drinkers, but from previous investigation it was obvious that one was more susceptible to alcohol than the other. Commercial 95% grain alcohol was diluted to about 20% with tap water and ingested in a period of 5 minutes or less, on an empty stomach unless otherwise stated. All alcohol doses are re-

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* Supported in part by a grant from the Rockefeller Fluid Research Fund of Stanford University School of Medicine.

[1] Mellanby, E., Med. Res. Council (Gr. Br.) Special Report Series No. 31, 1919.

[2] Mirsky, I. A., Piker, P., Rosenbaum, M., and Lederer, H., *Quart. J. Stud. Alcohol,* 1941, **2**, 35.

[3] Eggleton, M. G., *Brit. J. Psychol.,* 1941, **32**, 52.

corded as grams of 100% ethyl alcohol. Subsequent small doses required to maintain the blood concentration constant were diluted with milk to slow absorption. Samples of blood, urine, or saliva were collected at appropriate intervals, and alcohol determined by the method of Newman.[4] The degree of drunkenness was tested by means of a commercially available device whereby accuracy in aiming a gun at a moving target was automatically recorded, the test occupying about 8 minutes. Practice over a period of a week was sufficient to eliminate any increment in performance due to learning. A perfect score was 150; this was occasionally achieved by these subjects. Control runs varied less than 5 points.

Fig. 1 shows the results following ingestion of 0.8 g of alcohol per kg 2 hours after lunch by the more susceptible subject. The maximum of both alcohol concentration and decline in performance came at 1 hour. Subsequently, although the alcohol concentration did not change appreciably, by 2½ hours performance had returned to normal.

Fig. 2 represents the results when the less susceptible subject ingested a dose of 1.0 g per kg, and subsequently maintained his alcohol concentration constant over a period of 6 hours by means of

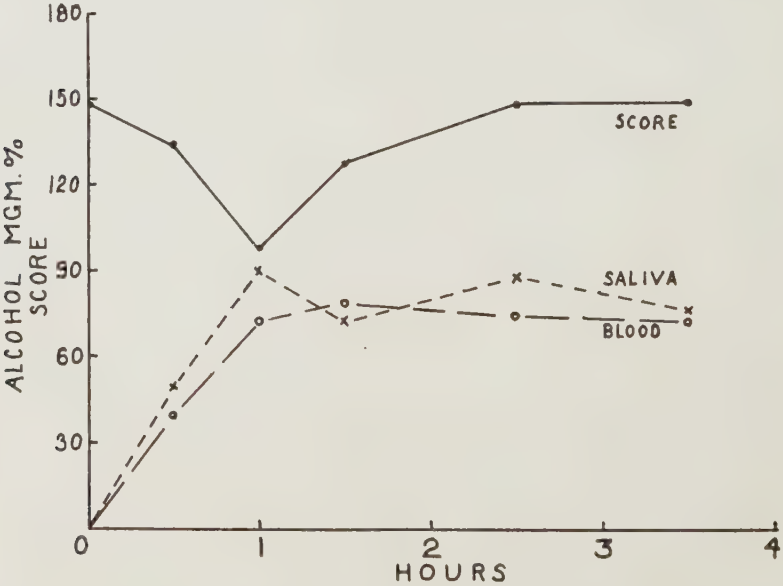

FIG. 1.

Alcohol concentration and test performance after ingestion of a single dose of 0.8 g of alcohol per kg body weight in a relatively susceptible subject.

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[4] Newman, H. W., *J. Pharm. and Exp. Therap.*, 1936, **56**, 278.

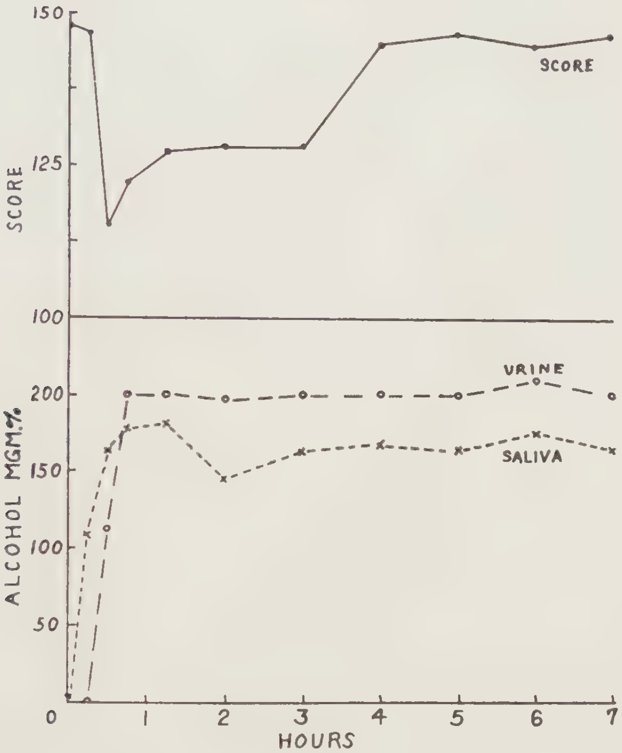

FIG. 2.

Alcohol concentration and test performance after ingestion of a dose of 1.0 g of alcohol per kg, followed by hourly doses of 8.0 g of alcohol diluted with milk in a relatively resistant subject.

hourly doses of 8.0 g of alcohol in milk. Maximal alcohol concentration and decline in performance came at 30 to 45 minutes. However, by the end of 4 hours, performance had returned to normal, in spite of the continued presence of a rather high alcohol concentration.

In Fig. 3 we see the results when the same subject raised his alcohol concentration slowly by ingesting 16.0 g of alcohol in milk hourly for 6 hours. It is striking that in no respect was performance affected by this slow increment of alcohol concentration.

Fig. 4 bears directly on the question of whether the important factor is the actual rate of increment, or the period of time elapsing during which "adaptation" to alcohol can take place. A small dose, 0.5 g per kg, was ingested by the less susceptible subject, resulting in the prompt attainment of a concentration insufficient to lower performance. This concentration was maintained for 4 hours by hourly ingestion of 8.0 g of alcohol. Then another dose equal to the

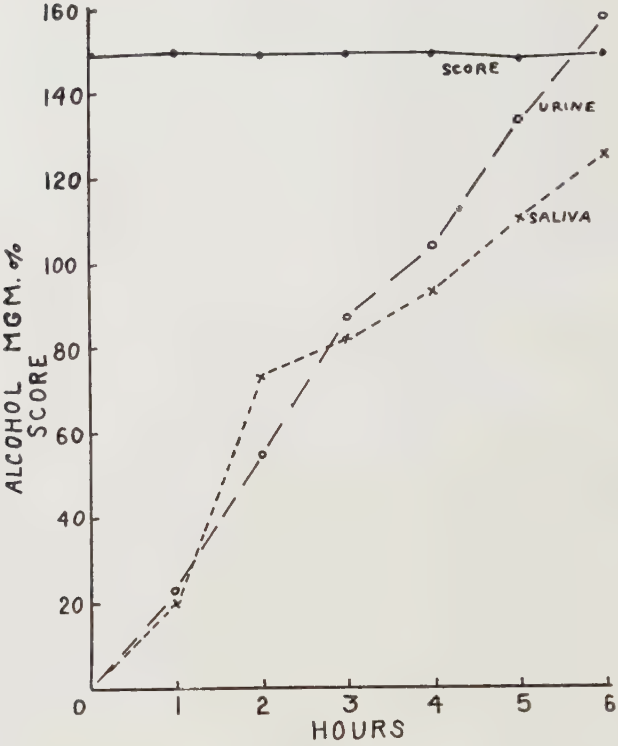

Fig. 3.

Alcohol concentration and test performance with ingestion of 16.0 g of alcohol in milk hourly in the resistant subject.

initial one was given, resulting in a rapid rise to a concentration which, when attained initially by the same subject in a previous experiment, shown in Fig. 2, had resulted in a considerable decrease in performance. Here, although the rate of increment was quite comparable to that in the previous experiment, apparently the 4-hour period at a lower alcohol concentration had been effective in "adapting" the nervous system, and no decrease in performance resulted.

Summary. The presence of alcohol in the body over a period of several hours effects a change in the response of the nervous system to alcohol, with the result that concentrations which initially produced drunkenness no longer are capable of showing this effect. Thus the effect of a given concentration of alcohol depends not only on its absolute value but also on how long a time it has been present in the body.

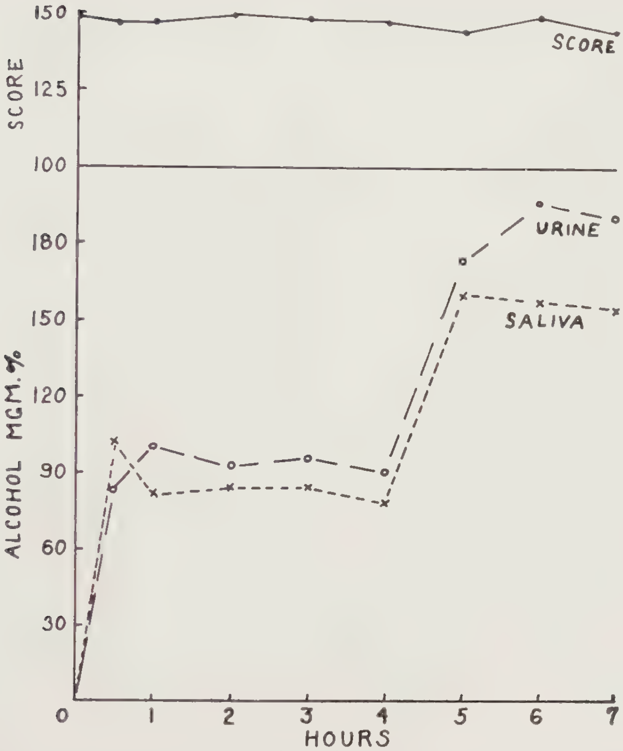

FIG. 4.

Alcohol concentration and test performance after 2 doses of 0.5 g of alcohol per kg separated by 4 hours. The alcohol concentration was maintained constant in the interval and after the second dose by hourly doses of 8.0 g of alcohol in milk.

13374

Possible Rôle of the Kidney in Regulation of Normal Blood Pressure in the Dog.

R. M. HOUSE AND G. E. WAKERLIN.

From the Department of Physiology, University of Illinois College of Medicine.

The well-known rôle of the Goldblatt kidney in the production and maintenance of one form of experimental hypertension[1] raises the question of a possible specific rôle for the kidney in the regulation of normal blood pressure. Wakerlin and Chobot[2] found no evidence that the renal pressor substance, renin, plays a rôle in the maintenance of normal blood pressure, although Page and Helmer[3] suggest that angiotonin which they consider to result from the interaction of renin and renin activator, may be a humoral means for the regulation of blood pressure. The possible rôle of the kidney in the regulation of normal blood pressure was studied by comparing the blood pressure responses of bilaterally nephrectomized and sham nephrectomized dogs to one of two conditions of vascular stress, *viz.,* bilateral splanchnicotomy and thoracic chordotomy.

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[1] Goldblatt, H., *Am. J. Clin. Path.*, 1940, **10**, 40.

[2] Wakerlin, G. E., and Chobot, G. R., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 331.

Dogs were anesthetized with 250 mg per kilo of sodium barbital intraperitoneally and blood pressure was recorded from the cannulated carotid or femoral artery by means of a mercury manometer. Nephrectomies and sham nephrectomies were performed by the retroperitoneal lumbar approach.

The splanchnicotomized group consisted of 24 dogs divided equally between nephrectomized and control animals. Both splanchnic nerves were isolated at the time of nephrectomy or sham nephrectomy. Three hours later bilateral splanchnicotomy was performed and blood pressure readings were continued for another 3 hours. Three hours were allowed to elapse before splanchnicotomy was performed on the theory that any humoral effect on blood pressure from the normal kidney might have been at least partially dissipated. Possibly relevant in this connection is the fact that the sensitivity of dogs to the acute pressor effect of renin administered intravenously frequently increases one to 3 hours[2] and 24 hours[4, 5] after nephrectomy.

In the 2 chordotomized groups the same procedures were followed except that instead of isolating the splanchnics, the spine and part of the laminae of the sixth thoracic vertebra were removed at the time of nephrectomy or sham nephrectomy, and in one of the groups 24 instead of 3 hours were allowed to elapse between nephrectomy or sham nephrectomy and chordotomy. A lifting ligature was placed under the exposed chord at the time of laminectomy and the wound temporarily sutured. Chordotomy was performed 3 hours later in one group of 18 dogs, equally divided between nephrectomized and sham nephrectomized animals. Following chordotomy, blood pressure readings were continued for 3 hours.

In the other group of 10 dogs, nephrectomy or sham nephrectomy,

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[3] Page, I. H., and Helmer, O. M., *J. Exp. Med.,* 1940, **71**, 29.

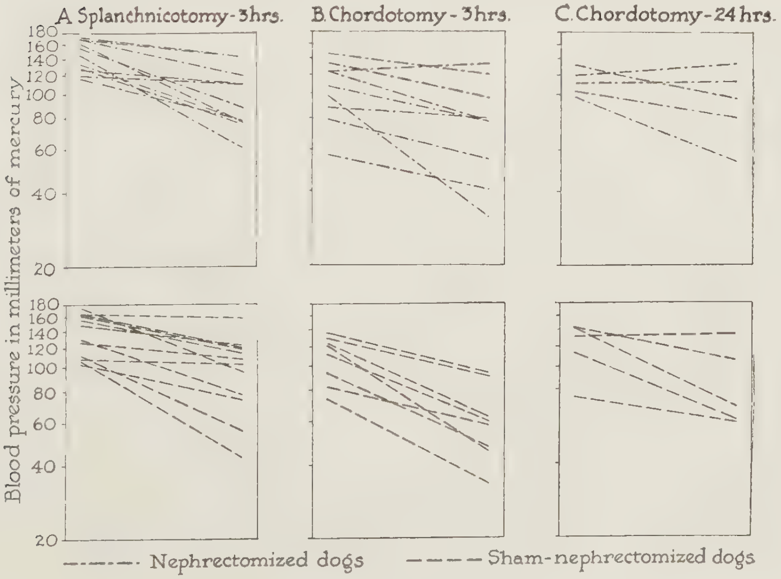
[4] Merrill, A., Williams, J. R., Jr., and Harrison, T. R., *Am. J. Med. Sci.,* 1938, **196**, 18.

[5] Tigerstedt, R., and Bergman, P. G., *Skand. Arch. Physiol.,* 1898, **8**, 223.

and exposure of the sixth thoracic segment of the spinal chord were carried out under morphine-pentobarbital anesthesia and under sterile conditions. These dogs were also equally divided between nephrectomized and control animals. Twenty-four hours later with the animals under sodium barbital anesthesia chordotomy was performed and the blood pressure recording continued for 3 hours.

The results showed no significant difference, confirmed by statistical analysis, using the "t" test, between the decreases in blood pressure in the nephrectomized and sham nephrectomized groups following splanchnicotomy or chordotomy. Thus the percentages of fall in blood pressure 3 hours after splanchnic section varied from 8 to 57, with an average of 33 for the nephrectomized group, and from 4 to 60 with an average of 28 for the control group. (Fig. 1, Col. A.) The percentages of fall in blood pressure 3 hours after chordotomy varied from 9 to 64 with an average of 26 for the nephrectomized group, and from 31 to 63 with an average of 45 for the sham nephrectomized group. (Fig. 1, Col. B.) The percentages of fall in blood pressure for the 24-hour chordotomy experiments ranged between 0 and 64 with an average of 21 for the nephrectomized animals, and between 0 and 52 with an average of 29 for the sham nephrectomized animals, demonstrating that the longer period for

Figure 1.
Effect of Splanchnicotomy and Chordotomy on Blood Pressures of Nephrectomized and Sham-nephrectomized Dogs



dissipation of a possible humoral effect from the kidney did not significantly alter the results. (Fig. 1, Col. C.)

Though these findings by no means rule out a specific rôle for the kidney in the regulation of normal blood pressure, they give no support to this possibility and add to the probability that renin, like epinephrine and vasopressin, does not exert a pressor effect under normal conditions.

Conclusions. (1) There was no significant difference between the decreases in blood pressure shown by nephrectomized and sham nephrectomized dogs following splanchnicotomy or chordotomy. (2) These results lend no support to the possibility that the kidney plays a specific rôle in the regulation of normal blood pressure in the dog.

13375

Physiology of Bacteria-Free Culture of *Trichomonas vaginalis:* VI. Effect of Female Sex Hormones on Population.*

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Exacerbations are frequently observed in cases of *Trichomonas vaginalis* vaginitis following menstruation or during pregnancy. In search of a possible explanation of this phenomenon Stein and Cope[1] examined the effect of progynon (Schering) upon contaminated cultures of *Trichomonas vaginalis* and concluded that "the addition of female sex hormone to culture medium stimulated the multiplication of *Trichomonas vaginalis in vitro.*" They also state[1] that "the sex hormone content of menstrual blood may be a more important factor in causing the postmenstrual flare of *Trichomonas vaginalis* vaginitis than the presence of blood serum and possibly also of tissue fragments."

The purpose of the present study was to reinvestigate the rôle of female sex hormones using a bacteria-free culture.

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* The authors are indebted to Dr. Ray E. Trussell, Dr. E. D. Plass and Dr. T. L. Jahn for much helpful criticism in the course of this study.

[1] Stein, I. F., and Cope, E. J., *Am. J. Obst. and Gynec.*, 1933, **25**, 819.

Solutions of theelol (Parke, Davis Co.), estradiol (Ciba), and estradiol-3-benzoate (Ciba) in 95% alcohol were prepared in 3 dilutions: estradiol-3-benzoate 0.01, 0.001, and 0.0001%; estradiol and theelol 0.1, 0.01, and 0.001%.[†] In each case 0.02 cc of hormone solution was added to 10.0 cc of sterile culture fluid by tuberculin syringe without the needle attached. The medium which was adjusted to pH 6.0 with N/1 HCl contained 5% human serum in Baltimore Biological Thioglycollate medium without glucose.

Ten cultures were employed for each dilution of the 3 hormones and compared with 10 control cultures which received 0.02 cc of 95% alcohol without the hormone. The inoculum consisted of 0.2 cc of culture fluid obtained by mixing several 4-day-old stock cultures. The incubation temperature was 35 ± 0.5°C. Populations per cubic millimeter were determined by hemocytometer count at 24-hour intervals until the concentration of organisms was demonstrated to have passed the maximum. The culture tubes were examined for evidence of gross contamination and checked further by streaking on chocolate agar plates. Contamination was demonstrated in 2 cultures; these results were excluded. Table I shows the content in gammas of hormone per tube of culture medium. The population data were taken from the first of 2 series of experiments with parallel results.

As a preliminary check on the effect of alcohol, batteries of 10 tubes with and without 0.2% alcohol were inoculated and the population increases compared in 2 series. Flagellate counts on 6 successive days demonstrated no significant difference.

Selection of the dosage of hormone to be used was based upon rat assays of vaginal discharge from women in the last month of pregnancy. The authors are indebted to Mr. Tom Hernandez of the Department of Zoology for performing these tests. Positive estrous smears were obtained in rats receiving 0.07 cc of vaginal discharge and negative smears from those receiving 0.05 cc. It was, therefore, decided that the hormone concentration employed in the cultures should range above and below the value required to produce such an estrous response since it would be comparable to the concentration encountered in the vaginal discharges of pregnant women.

Reference to Table I will reveal that in no case did the experimental cultures with hormone differ significantly from the control cultures. It is, therefore, concluded that under the conditions of these experi-

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[†] Professor Emil Witschi of the Department of Zoology kindly supplied the hormone solutions which were used and made possible a portion of the work which was carried out in his laboratory.

TABLE I.
Effect of Female Sex Hormones on Population Increase of *Trichomonas vaginalis.*

		Avg population per mm^3 Day examined					
Hormone	Gammas per 10 cc of culture medium	1	2	3	4	5	6
Estradiol-3-benzoate	2.100	29	66	132	221	287	282
	0.210	30	63	119	198	279	272
	0.021	29	76	131	214	271	279
Control		26	67	125	203	276	273
Estradiol	21.000	38	94	190	251	327	322
	2.100	42	58	219	305	329	318
	0.210	33	97	199	306	329	326
Control		36	100	219	311	342	320
Theelol	21.000	36	79	165	231	327	315
	2.100	36	90	181	226	321	318
	0.210	34	85	170	228	324	314
Control		36	100	219	311	342	320

ments the female sex hormones here employed have no effect upon increase in population of bacteria-free cultures of *Trichomonas vaginalis.*

13376 P

Formation of an Antirachitic Product from Cholesteryl Sulfates.

Albert E. Sobel and Sidney P. Gottfried. (Introduced by B. Kramer.)

From the Pediatric Research Laboratory, Jewish Hospital of Brooklyn.

This communication deals with a report of some of the conditions under which an antirachitically active product is obtained from some of the new cholesteryl sulfates recently prepared.[1]

Eck and Thomas reported the formation of an antirachitic product on heating ammonium or potassium cholesteryl sulfate at 220°-250° for 30 minutes. The yields were low, approximately 0.09 I.U. per mg of the ether extract of the heated product.[2] At first the conditions of Eck and Thomas were repeated with slight modifications using the newly prepared barium, pyridonium, silver, as well as the previously known potassium cholesteryl sulfate.[3, 4] The results are presented in Table I. The yield of antirachitically active product (in terms of international rat units) was much higher in the case of the barium, pyridonium, and silver salts than in the case of the potassium salt. Our potassium salt gave a lower activity than that reported by Eck and Thomas, but of the same order of magnitude. When any of the salts were heated at their respective decomposition temperatures, no antirachitically active products were obtained.

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[1] Sobel, A. E., and Spoerri, P. E., *J. Am. Chem. Soc.*, 1941, **63**, 1259.

[2] Eck, J. C., and Thomas, B. H., *J. Biol. Chem.*, 1937, **119**, 631.

In the next experiment the salts were suspended in tetralin and oxygen bubbled through. Under the conditions stated in Table I, the barium cholesteryl sulfate gave an antirachitically active product. All the other salts were negative in the amounts tested. When air was substituted for oxygen, none of the salts yielded an active fraction.

TABLE I.

Cholesteryl sulfate	No. of rats	Conditions	I.U. of vitamin D per mg of petroleum ether soluble fraction
Barium, lead, silver, potassium	2	Heated in air at their respective decomposition temp. 10 to 15 min.	Negative (fed 70 to 80 mg 10 days)
Barium	5	Heated in air at 250° 10 to 15 min.	.50 per mg cholesterol in starting material
Pyridonium	7	”	1.20
Silver	6	”	0.99
Potassium	5	”	.02 per mg cholesterol in starting material
Barium	5	Heated in tetralin at 200° 15 to 30 min. while bubbling through oxygen	1.11
Pyridonium, lead, silver, potassium,	2	”	Negative (fed 6 to 12 mg 5 days)
Pyridonium, barium, silver, potassium, lead	6 2	Heated in tetralin at 200° 15 to 30 min. but bubbled through air	Negative (fed 16 to 32 mg 5 days)
Barium	5	Heated in an evacuated tube at 250° 15 min.	1.6
Silver	5	”	2.8
Pyridonium	2	”	Negative (fed 23 mg 5 days)
Potassium	4	”	Negative (fed 50 to 200 mg 10 days)

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[3] Mandel, I. A., and Neuberg, C., *Biochem. Z.*, 1915, **71**, 186.

[4] Natelson, S., Sobel, A. E., and Kramer, B., *J. Biol. Chem.*, 1934, **105**, 761.

Since we desired to study further the effect of oxygen on antirachitic activation, the barium, pyridonium, and silver salts were heated at 250° in an evacuated tube. The results are tabulated in Table I. Both the barium and silver salts gave a positive result. The pyridonium and potassium salts were inactive on repeated attempts.

It thus appears that oxygen is necessary for the antirachitic activation of the potassium and pyridonium salts but not for the barium and silver salts. It also appears that the nature of the cation plays an important rôle in the antirachitic activation under a given set of conditions.

Experimental. The cholesteryl sulfates employed were prepared according to the procedure of Sobel and Spoerri.[1] When the salts were heated in the dry state, the residue was extracted with petroleum ether (B.P. 35°-60°). When the salts were heated in tetralin, the latter was first evaporated under vacuum, keeping the temperature under 125°, and the residue extracted with petroleum ether. In both cases, the petroleum ether soluble residue was dissolved in 10 cc of Vitamin D-free maize oil and fed to the rachitic rats.

The method of bioassay was similar to that by Natelson and Sobel.[5] By means of a pipette, 0.1 cc daily feedings of maize oil solutions of the petroleum ether extract were carried out for 5 days. At the same time pure maize oil and maize oil solutions containing 3 units of Vitamin D in 0.1 cc were fed daily to several control rats. The rachitic rats receiving pure maize oil showed severe rickets with no healing. The rats that were fed a total of 15 International Units of Vitamin D in 5 days showed a typical 2 plus healing. The conversion of healing more or less than 2 plus in terms of 2 plus healing was accomplished according to the procedure of Bills, *et al.*[6] In other studies carried out in this laboratory, we have found the method of Bills to be quite reliable.

In preliminary bioassays which preceded the assays reported in the table, X-rays of the rats were taken at the end of 5, 7, and 10 days in order to obtain the approximate range for a more exact bioassay. During this period daily feedings of 0.1 cc of maize containing the petroleum ether extract took place.

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[5] Natelson, S., and Sobel, A. E., *J. Biol. Chem.*, 1935, **109**, 687.

[6] Bills, C. E., Honeywell, E. M., Wirick, A. M., and Nussmeier, M., *J. Biol. Chem.*, 1931, **90**, 619.

13377

Effects of Soft X-rays on Urease and Catalase.

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Introduction. Many investigators have reported that X-rays had little or no effect on enzymes. Scott[1] concluded that enzymes are inactivated only when the dose of X-rays is enormous. On the other hand it is well known that cell division is inhibited by relatively small doses. Most of the work prior to 1937 was carried out on impure enzyme solutions and the results are inconsistent with a recent report by Dale[2] to the effect that polyphenol oxidase and carboxypeptidase are inactivated by hard X-rays in dilute solution of the pure enzymes.

The work described below was designed to answer the following questions: (1) To what extent is the susceptibility of enzymes to X-rays dependent upon the purity of the preparation? (2) Does the susceptibility vary with the concentration of the enzyme? The answer to these questions is necessary in order to evaluate the possible participation of enzyme destruction in the observed effects of X-rays on cells.

It can be said at the outset that the results show that crystalline preparations of urease and catalase are more easily inactivated than impure preparations of the same enzymes and that the susceptibility increases with dilution.

Procedure. Source of Radiation. The radiation was supplied by a gas X-ray tube having a copper target and window made of aluminum and cellophane. It was operated at 30 peak kv and 10 ma.[3] The shortest wavelength emitted was about 0.4 Ångström units, while the most intense part of the radiation was the K alpha line of copper which has a wavelength of 1.54 Ångströms. This region of the X-ray spectrum is of considerably longer wavelength than the one used in medical radiography and its energy is absorbed to a greater degree by matter. For this reason, one would expect that, having two sources each emitting the same energy, one in the long wavelength region and one in the short wavelength region, any chemical or biological change due to X-rays would be more pronounced when caused by the long wavelength X-rays since more of the incident energy would remain behind in the substance irradiated. Thus it may happen that effects which are easily produced with long wavelength radiation may seem impossible of production with radiations used in medical radiography.

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[1] Scott, C. M., Medical Research Council, Great Britain, 1937, Special Series Report No. 223.

[2] Dale, W. M., *Biochem. J.*, 1940, **34**, 1367.

[3] Kersten, H., *Radiol.*, 1934, **23**, 60.

The enzyme solutions were irradiated in small glass cups placed in a special holder within a Dewar flask filled with crushed ice. The cup was covered with a cellophane window and the window exposed to the radiation at a distance of 3 cm from the focal spot of the target. The temperature within the irradiation chamber was 6-8°C as determined by a thermocouple arrangement. Five cubic centimeters of solution were irradiated at a time. In all cases the irradiated solutions were tested as soon as possible after irradiation and in each case the irradiated sample was measured against a control from the same dilution kept at the same temperature as the test sample.

Enzyme Preparation and Tests. Crude Urease. Ten grams of Arlco jack-bean meal were thoroughly mixed with 50 cc of distilled water in a beaker. The mixture was allowed to stand in the cold room at 4°C for 24 hours. The digest was then centrifuged from the debris at 4000 rpm in the cold. The supernatant fluid was then diluted to the desired concentration with distilled water. No stabilizers such as bisulfite were used. The solution was kept at 4°C when not in use. Fresh preparations were made daily.

Crystalline Urease. Samples of crystalline urease were prepared according to the method of Sumner[4] and recrystallized according to the method of Sumner and Hand.[5] Saturated suspensions in distilled water were stored at 4°C and diluted to a suitable concentration before use. The recrystallized samples of urease usually had an activity of 50,000-80,000 units per gram.

Method of Testing Urease Activity. Urease activity was measured by Sizer's method[6] of measurement of the rate of liberation of CO_2 from urea-phosphate solution.

Crude Catalase. The crude dioxane filtrates from beef liver and mother liquor immediately prior to dialysis for crystallization in the method of Sumner and Dounce[7] were used as "crude" catalase.

Crystalline Catalase. Crystalline catalase was prepared according to the method of Sumner and Dounce. The crystalline preparations usually had a Kat. f. = 17,000-37,000.

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[4] Sumner, J. B., *J. Biol. Chem.*, 1926, **69**, 435.

[5] Sumner, J. B., and Hand, D. B., *J. Biol. Chem.*, 1928, **76**, 149.

[6] Sizer, I. W., *J. Gen. Physiol.*, 1939, **22**, 719.

[7] Sumner, J. B., and Dounce, A. L., *J. Biol. Chem.*, 1939, **127**, 439.

Method of Testing Catalase Activity. The test solution and all the reagents were cooled to 4°C and maintained at that temperature during the test. 0.5-1.0 cc of catalase solution was added to 35 cc 0.02 N H_2O_2 and 10 cc of M/15 phosphate buffer ($pH = 6.8$) and 5 cc distilled water. After a specified time 5 cc of 10 N H_2SO_4 were added and the residual peroxide was titrated with 0.02 N $KMnO_4$.

Results. Urease. Table I shows that irradiation for 20 minutes of urease solutions *below certain concentrations* resulted in inactivation, the percentage of which (1) increased with dilution for all crystalline preparations but (2) was relatively greater, *at the same concentrations,* for solutions made from highly active than from partially inactive material.

TABLE I.

Effect of Dilution of Crystalline Urease Solutions on Sensitivity to Soft X-ray Irradiation for 20 Minutes.

Activity before irradiation *Units per cc	Activity after irradiation Units per cc	% inactivation
†31	31	0
15.5	15.5	0
3.1	1.24	60
1.5	0.30	80
‡12	12	0
6.0	6.0	0
1.2	0.79	34
0.6	0.22	63

*1 urease unit = amount of enzyme which will liberate 1 mg ammonia N in 5 min. at 20°C.

†Prepared from crystals having 87,000 units per g.

‡Prepared from crystals having 28,000 units per g.

Urease solutions containing 1.5 units per cc were prepared from crude preparations and from crystals of high activity (87,000 units per gram). Eighty percent of the crystalline preparation was inactivated in 20 minutes whereas it required 70 minutes to produce the same degree of inactivation in the crude enzyme (Fig. 1). A similar difference in the reaction of crude and crystalline urease to oxidation and reduction reagents has been observed by Sizer and Tytell.[8] Crystalline urease responded to the oxidation and reduction reagents whereas the crude preparations were unaffected.

Catalase. The results show that catalase is less radiosensitive than urease to soft X-rays. Crude preparations did not show inactivation in either dilute or concentrated solution even after 8 hours' irradiation.

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[8] Sizer, I. W., and Tytell, A. A., *J. Biol. Chem.*, 1941, **138**, 631.

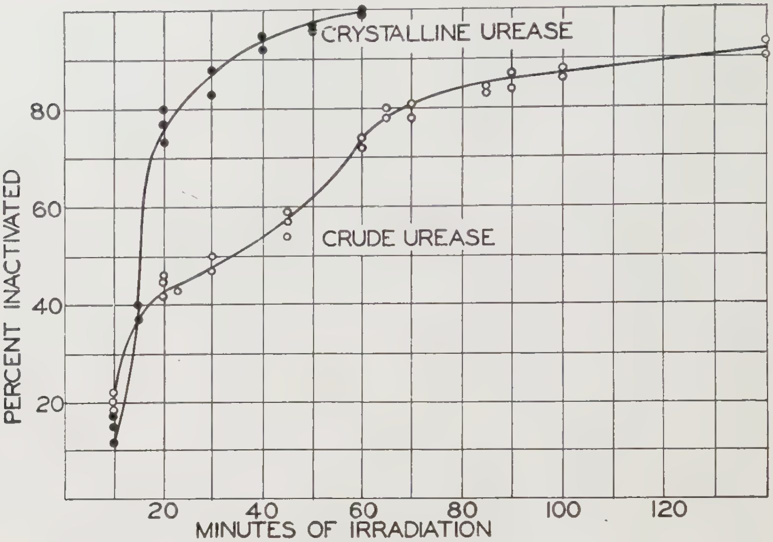

FIG. 1.

Time-inactivation curve for crude and crystalline urease solutions irradiated with soft X-rays.

Crystalline preparations were partially inactivated by large doses of radiation. Preparations made from crystals of high catalase activity were more easily inactivated than those made from crystals exhibiting lower activity (Table II). Sensitivity increased with dilution (Fig. 2). Except for the fact that catalase was less sensitive to X-rays, the results are similar to those obtained with urease.

Discussion. As a working hypothesis to explain the insensitivity of concentrated and impure enzyme solutions it may be reasonable to assume that the groups sensitive to X-rays are reversibly combined with other groups whereby they become stabilized. If the group-

TABLE II.

Sensitivity of Solutions of Crystalline Catalase Preparations of Varying Activities to Soft X ray Irradiation for 4 Hours.

* Kat. f.	Dry solids of irradiated solutions, mg per cc	% inactivation
17,228	2.8†	0.0
27,337	3.1†	18.0
36,320	3.3†	23.0
36,320	0.66‡	52.5

*Kat. f. (Katalase fähigheit) is the ratio of k, catalytic activity expressed in terms of the monomolecular rate constant, to the total enzyme solids in reaction mixture. Kat. f. was always determined on the freshly prepared crystals and suitable dilutions made for test.

†These solutions were irradiated at a dilution of 1:25 by volume of the saturated preparation. Concentrated preparation in Fig. 2 was prepared in this manner.

‡This solution was prepared by diluting the 1:25 solution 5 times by volume, *i. e.,* final dilution 1:125.

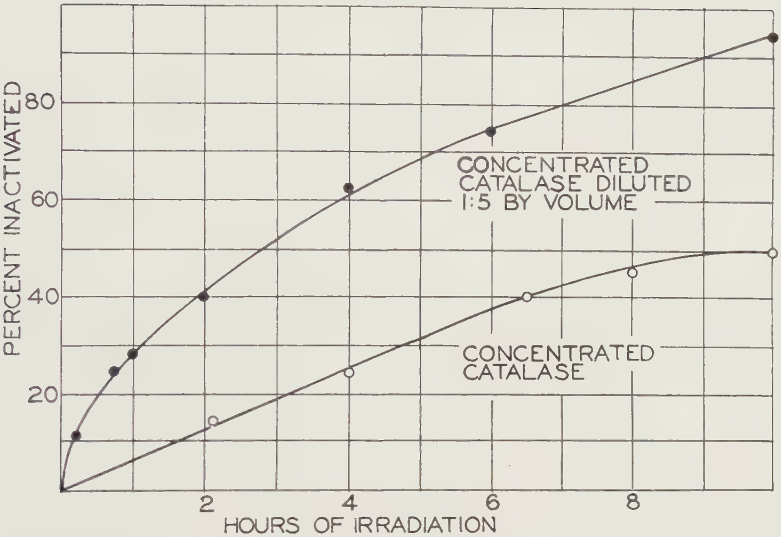

Fig. 2.

Effect of dilution on the time-inactivation curve of crystalline catalase solutions irradiated with soft X-rays.

ings on the enzyme are susceptible to X-rays only when dissociated, then this is consistent with the observed enhancement of sensitivity on dilution.

Estimation of the sensitivity of an enzyme within a cell to X-rays would then be contingent upon an understanding not only of the concentration but of the extent to which the enzyme is freely dissociated in the cell.

Summary. 1. The effects of soft X-rays on crude urease, crystalline urease, crude catalase, and crystalline catalase are described. 2. The sensitivity of the enzymes to the radiation is dependent upon purity and concentration. 3. Results are given which are in accordance with the observations of Dale on the effects of hard X-rays on polyphenol oxidase and carboxypolypeptidase.

13378

Tryptophane Requirement of the Chick.

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In studies on the amino acid requirements of chicks it has been found necessary to ascertain the quantitative tryptophane requirement as one of the preliminaries to working on other amino acids. This is due to the facts (1) that acid-hydrolysed proteins, from which it is desired to remove certain amino acids chemically, have suffered a practically complete loss of tryptophane, and (2) certain proteins of interest because of natural deficiencies are also low in tryptophane content.

The chick diet used was a modification of a simplified chick diet which has been developed especially for work with proteins. The diet contained, per 100 g: casein, washed or acid hydrolysed, 20; gelatin, 10; calcium gluconate, 5; gum arabic, 5; tricalcium phosphate, 2; dipotassium phosphate, 0.5; potassium chloride, 0.5; manganese sulphate, 0.1; magnesium sulphate, 0.2; sodium silicate, 0.2; sodium chloride, 0.5; choline chloride, 0.3; ox bile salts, 0.5; wheat germ oil, 3; cod liver oil, 1; cotton pulp, 5 g. The vitamin B complex was added in the form of thiamin, 1 mg; riboflavin, 1 mg; pyridoxine, 1 mg; calcium pantothenate, 3 mg. A hexane extract of alfalfa equivalent to 2 gm was added as a source of vitamin K. A small quantity of a special mineral mixture was also added to provide iron, 1 mg; copper, 0.5 mg; zinc, 0.5 mg; cobalt, .02 mg; and iodine, 0.4 mg. An extract of brewers' yeast prepared by extraction with 50% aqueous methanol was concentrated, made slightly acid with acetic acid, and clarified by chilling and filtration. This was used at a level equivalent to 5%. Cerelose (glucose) was then added to complete the balance of 100 g. The principal features of this diet are the elimination of cereals and their by-products, and the reduction of protein sources to casein and gelatin.

An experiment was conducted to determine whether casein at a level of 20% would supply sufficient tryptophane for optimum growth of chicks on this diet. Chicks were first reared for 1 to 1½ weeks on a practical diet, then selected for uniform weight and condition, divided into groups of equivalent average weight, and given the experimental diets.

Pure l-tryptophane was added to the diet at various levels and the

TABLE I.
Growth-promoting Effects of l-tryptophane Additions to a Chick Diet Containing 20% Whole Casein.

l-tryptophane added, %	Avg gain per chick, g	Gain per day, %
0.0	43.3	6.7
0.16	46.0	7.0
0.26	44.2	6.8
0.36	46.0	7.0

Each group contained 5 chicks with an average starting weight of 50 g. The experiment lasted 9 days.

growth rate of the chicks determined at frequent intervals for 6 to 9 days. Results of the first experiment are given in Table I. It is evident from this experiment that additions of l-tryptophane did not increase the rate of growth of the chicks. Recent tables of amino acid composition of proteins[1] give a value of 2.2% for the tryptophane content of casein and 0.0% for that of gelatin. The tryptophane content of the diet, therefore, could not be appreciably more than 0.44%. This appeared to be sufficient for a good rate of growth. The rate of growth shown by chicks on the experimental diet was approximately equal to that which would be made by the same chicks on the practical rearing diet.

Other experiments were planned to investigate lower tryptophane levels. Casein was completely hydrolysed by means of strong sulphuric acid, the acid removed by barium hydroxide, and the hydrolysate concentrated under reduced pressure, decolorized with activated

TABLE II.
Growth-promoting Effect of l-tryptophane Additions to a Tryptophane-deficient Chick Diet Containing 20% Acid-hydrolysed Casein.

	Exp. 1*		Exp. 2†		Exp. 3‡	
l-tryptophane added %	Avg gain per chick, g	Gain per day, %	Avg gain per chick, g	Gain per day, %	Avg gain per chick, g	Gain per day, %
.0	— 6.4	—2.4	— 7.6	—2.2		
.2	+12.0	+3.7	+14.3	+3.3		
.3					+19.7	+3.3
.4			+19.6	+4.6	+26.1	+4.2
.5					+33.3	+5.1
.6			+25.3	+5.3	+33.9	+5.2

*Each group contained 4 chicks with an average starting weight of 48 g. The experiment lasted 6 days.

†Each group contained 5 chicks with an average starting weight of 47 g. The experiment lasted 8 days.

‡Each group contained 5 chicks with an average starting weight of 65 g. The experiment lasted 8 days.

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[1] Calvery, H. O., *Chemistry of the Amino Acids and Proteins* (edited by C. L. A. Schmidt), Charles C. Thomas, publisher, Baltimore, Maryland, first ed., p. 217.

carbon, mixed with cotton pulp and dried in a current of warm air. Colorimetric tests for tryptophane (Hopkins-Cole, Rosenheim) were negative. The mixture was placed in the diet in lieu of the whole casein and cotton pulp. Results with this diet are given in Table II.

It will be seen from Table II that chicks given only the basal diet suffered a distinct loss in weight. This loss was clearly indicated within the first 24 hours of the experiments. Evidently, there was no appreciable tryptophane reserve. On the other hand, growth took place in the groups receiving as little as 0.2% l-tryptophane in the diet. The tendency of the latter groups to grow was also clearly indicated within 24 hours in most cases, although there was some evidence that chicks did not become fully accustomed to the new diet in this length of time. The magnitude of the differences in growth rates would indicate that the basal diet was practically free of tryptophane but that no other serious deficiency existed.

Maximum rate of growth was attained at 0.5 to 0.6% of added l-tryptophane. This rate was substantially less than that of chicks on the whole casein diets. Such differences in growth-promoting values of whole as compared to hydrolysed casein for the chick have been previously reported[2, 3] even in the case of enzymatic hydrolysis,[3] and are, therefore, to be expected.

Summary. The l-tryptophane requirement for optimum growth of the young chick is approximately 0.5% of the diet.

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Similarities and Possible Relationships Among Viruses of Psittacosis, Meningopneumonitis, and Lymphogranuloma Venereum.

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Findlay, Mackenzie and MacCallum[1] have described a developmental cycle for the virus of lymphogranuloma venereum in the brains of infected mice and have pointed out that the developmental

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[2] Klose, A. A., Stokstad, E. L. R., and Almquist, H. J., *J. Biol. Chem.*, 1938, **123**, 691.

[3] Stokstad, E. L. R., *Poultry Science*, 1940, **19**, 42.

[1] Findlay, G. M., Mackenzie, R. D., and MacCallum, F. O., *Nature*, 1938, **141**, 877; *Trans. Roy. Soc. Trop. Med. and Hyg.*, 1938, **32**, 183.

forms present many analogies to those exhibited by the causal agent of psittacosis.[2, 3] More recently Rake, Jones and Shaffer[4] have studied the developmental cycle of the virus of lymphogranuloma venereum in the yolk-sac cells of the developing chicken embryo and have pointed out the quite amazing similarity in morphology and staining reaction between the respective developmental forms in the two viruses.

The viruses of lymphogranuloma venereum, psittacosis, meningopneumonitis,[5] and a virus causing atypical pneumonia in human beings[6] all produce meningitis after intracerebral inoculation, and pneumonia after intranasal inoculation in mice, and the lesions thus produced by each of the viruses are practically indistinguishable grossly or microscopically. After intradermal or subcutaneous injection these 4 agents produce granulomatous infiltrations in the skin of experimental animals.

Since the development of complement fixation tests for lymphogranuloma venereum,[7] and psittacosis, fixation with sera from venereally exposed but clinically non-lymphogranulomatous persons and certain lymphogranuloma venereum antigens has been observed.[8] False positive complement fixation reactions between psittacosis antigen and sera from syphilitic persons have also been reported.[9, 10] It was noted particularly[8] that as far as complement fixation between the lymphogranuloma antigen and the sera of venereally exposed persons was concerned, there was no correlation with the results of the Wassermann test. Persons with gonorrhea who had a negative Wassermann reaction gave positive lymphogranuloma fixation in as high a proportion as did syphilitics. The possibility that subclinical infection with the virus of lymphogranuloma venereum might be the common factor in these cross reactions, and the other similarities of the viruses under consideration as noted above, led us to investigate the serological relationship of these viruses.

On investigation, cross-reactions between the 3 viruses by comple-

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[2] Bedson, S. P., and Bland, J. O. W., *Brit. J. Exp. Path.*, 1932, **13**, 461; 1933, **14**, 267; 1934, **15**, 243.

[3] Yanamura, H. Y., and Meyer, K. F., *J. Inf. Dis.*, 1941, **68**, 1.

[4] Rake, G., Jones, H. P., and Shaffer, M. F., *Am. J. Path.*, 1941, **17**, 460.

[5] Francis, T., Jr., and Magill, T. P., *J. Exp. Med.*, 1938, **68**, 147.

[6] Eaton, M. D., Beck, M. D., and Pearson, H. E., *J. Exp. Med.*, 1941, **73**, 641.

[7] McKee, C. M., Rake, G., and Shaffer, M. F., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 410.

[8] Shaffer, M. F., Rake, G., Grace, A. W., McKee, C. M., and Jones, H. P., *Am. J. Syph., Gon. and Ven. Dis.*, in press.

[9] Bedson, S. P., *Lancet*, 1935, **2**, 1277.

[10] Meyer, K. F., and Eddie, B., *J. Inf. Dis.*, 1939, **65**, 225.

TABLE I.

Cross Reaction in Complement Fixation Tests with Sera and Antigens of Psittacosis, Meningopneumonitis and Lymphogranuloma Venereum.

Serum	Case	Complement fixation titre with antigen from virus of		
		Psittacosis	Meningo-pneu-monitis	Lympho-granuloma venereum
L.G.V. No. 9	Lymphogranuloma venereum		16	>240
L.G.V. No. 31	,, ,,		8	120
L.G.V. No. 45	,, ,,		4	>120
L.G.V. No. 51	,, ,,	32		>120
L.G.V. No. 60	,, ,,	256		>120
L.G.V. No. 66	,, ,,	128		120
Ps. 7	,, ,,	+		>240
Ps. 11	,, ,,	++		>240
Rs	,, ,,		8	16
Pu	,, ,,		32	32
A.R. 11	Psittacosis	++		480*
A.R. 13	,,	++		240*
A.R. 18	,,	++		120*
Ps. 1	,, (Pigeon)	+		< 15
2	,, ,,	++		480
3	,, ,,	+		120
4	,,	+		>240
5	,,	++		480
8	,,	++		>1920
9	,,	++		240
6	Pneumonitis	+		120
B†	,,	256	32	64
P†	,,	64	16	32*
Da	,,	32	8	30
Gu	,,		8	30
Rod.	,,	4	8	6
MG.	,,	256	16	32
LC.	,,		32	4
St‡	,,		16	16
Mi	,,		64	32
Mey†	,,		8	32
Jo‡	,,		64	16
Ma†	,,		8	8
Ra‡	,,		16	32
Gr†	,,		8	8
W.P.M.†	Contact with pneumonitis		0	0
M.D.E.‡	,, ,, ,,		0	0
O.B.†	,, ,, ,,		0	0

*Fixation also with soluble antigen of lymphogranuloma venereum.
†Frei test positive.
‡Frei test negative.
+ positive.
++ strongly positive.

ment fixation have been found. Thus it will be seen from Table I that sera from cases of lymphogranuloma venereum fix not only with their homologous antigen, but also with antigens prepared from the viruses of psittacosis or meningopneumonitis; sera from cases of psittacosis fix not only with homologous antigen but also in high dilution with the antigen from lymphogranuloma venereum; while sera from cases of atypical pneumonia fix not only the meningopneumonitis antigen but also with antigens from lymphogranuloma venereum or psittacosis. None of the antigens gave fixation with normal human sera. The tests with psittacosis antigen on sera furnished by us were kindly done by Dr. K. F. Meyer at the laboratories of the George Williams Hooper Foundation, San Francisco. The psittacosis sera tested by us were received through the kindness of Dr. Meyer. More detailed studies with sera of immunized animals are being done in order to determine the exact nature of the cross reaction. The tests suggest the presence of a common antigenic factor in the viruses of psittacosis, meningopneumonitis, and lymphogranuloma venereum, but the possibility of cross reactions between these antigens and other unrelated viruses or microörganisms has not been completely excluded at the present stage of the work.

As is also shown in Table I, preliminary tests with soluble antigen prepared from virus of lymphogranuloma venereum[11] have shown that the heterologous fixation can be produced with this antigen. Whether it is responsible for all of the heterologous reaction remains to be investigated. Moreover, preliminary Frei tests carried out with the lymphogranuloma venereum yolk-sac antigen (Lygranum) have shown that in 5 out of 8 cases of atypical pneumonia a positive skin reaction could be elicited. Thus the antigen responsible for this reaction would seem to be either the same as that producing the cross reaction in the complement fixation test or another antigen common to this group of viruses.

Experiments to test the relationship by another method, namely, active immunity tests in mice, have shown that the viruses of meningopneumonitis and lymphogranuloma venereum do produce a reciprocal partial cross immunity.[12] Preliminary tests at New Brunswick suggest that some cross-relationship may exist between the viruses of lymphogranuloma venereum and meningopneumonitis as far as passive immunity is concerned.

From the fact that the viruses of psittacosis, meningopneumonitis, and lymphogranuloma venereum share similar morphology, developmental forms, and tinctorial characteristics, as well as common or closely related antigenic constituents, it is apparent that differentiation of these viruses must be made on the basis of source, quantitative antigenic differences, and a comparison of the differences in their pathogenicity for experimental animals.

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[11] Rake, G., Shaffer, M. F., Jones, H. P., and McKee, C. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 300.

[12] Eaton, M. D., Martin, W. P., and Beck, M. D., *J. Exp. Med.*, to be published.

13380

Influence of Traumatic Shock on Blood Sugar of Adrenalectomized Rats Treated with Adrenal Cortical Extract.*

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In a previous communication,[1] we called attention to the fact that local trauma exerts a pronounced influence on the sugar metabolism of tissues. We found that the venous blood coming from a damaged area is always much poorer in glucose than is the venous blood of a non-traumatized area in the same animal. This appeared all the more surprising since the systemic blood sugar is usually very high in traumatic shock. Further experiments showed that in adrenalectomized animals, whose resistance is low, traumatic shock is not accompanied by a rise in the systemic blood sugar, and indeed often causes hypoglycemia. These observations led us to conclude that disturbances in carbohydrate metabolism play a more important rôle in the pathogenesis of shock than is generally believed. It became evident, furthermore, that the adrenal cortical hormones exert a pronounced influence on sugar metabolism during the shock syndrome.

It is well known that adrenal extracts with corticoid hormonal activity[†] do not significantly influence the blood sugar of normal animals, although they are highly active in restoring to the normal level both the hypoglycemia of partially hepatectomized or insulin-treated and the hyperglycemia of adrenalin-treated animals.[2,3] In these cases, as well as in the hypoglycemia caused by adrenal insufficiency, the corticoid hormones appear to be stabilizers of the blood sugar, merely maintaining the glucose concentration at the normal level.

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[*] The expenses of this investigation were defrayed through a grant received from the National Research Council of Canada. The authors are particularly indebted to Dr. A. D. Odell of Charles E. Frosst & Co. who supplied the adrenal cortical extract used in these experiments.

[1] Selye, Hans, and Dosne, Christiane, PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 143.

[†] The term ''corticoid activity'' instead of adrenal cortical hormone-like activity is used here in agreement with the recently introduced terminology of the steroid hormone actions.[4]

[2] Selye, Hans, and Dosne, Christiane, PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 580.

[3] Selye, Hans, and Dosne, Christiane, *Am. J. Physiol.*, 1940, **128**, 729.

[4] Selye, Hans, *Nature*, 1941, **148**, 84.

It appeared of interest, therefore, to determine the possible rôle played by the adrenal cortex in the production of the characteristic shock hyperglycemia.

The following experiment appears to throw some light on this problem. Eighteen adult male albino rats weighing 138 g on the average, were adrenalectomized. During the 3 days following the operation, they were given "Purina" *ad lib.* and 0.9% NaCl solution instead of drinking water. On the third day, the saline solution was replaced by tap water and the animals were fasted for 16 hours. At the end of this period, the blood sugar of these animals as well as that of 6 intact but similarly fasted controls was determined (tail blood, Somogyi-Hartman-Schaffer method). After this the rats were subdivided into 4 groups of 6, 3 of these groups consisted of adrenalectomized and one of intact animals. In 2 groups of adrenalectomized animals, surgical shock was produced by crushing the abdominal muscles, the stomach and the cecum with a hemostat. Care was taken to produce the same degree of trauma in each case. One of these 2 groups, as well as the intact and adrenalectomized but not traumatized animals, were treated with 3 ml of an adrenal-cortical extract containing 1 Selye-Schenker[5] unit in 0.01 ml. This amount was administered subcutaneously in three 1 ml doses, the first injection being made 30 minutes before crushing the tissues in order to ascertain that a sufficient amount of corticoid hormone would be present in the circulation at the time of trauma. All animals were killed 3½ hours after the first injection and the blood was collected from the transcised jugular vein and carotid artery. Table I summarizes the results of the blood sugar and hemoglobin determina-

TABLE I.
Effect of Corticoid Extract on Traumatic Shock in Adrenalectomized Rats.

Treatment	Adrenalectomized and corticoid-treated	Adrenalectomized and traumatized	Adrenalectomized, traumatized and corticoid-treated	Corticoid-treated intact
Body weight	138 (128-151)	139 (125-152)	138 (124-153)	138 (128-148)
Hemoglobin	11.6 (11.0-12.5)	17.5 (16.6-18.4)	14.6 (14.0-15.2)	8.6 (8.1-9.2)
Blood sugar (before)	63 (54-71)	64 (58-71)	63 (54-71)	77 (67-92)
Blood sugar (after)	80 (75-83)	59 (50-67)	108 (83-124)	76 (67-83)

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[5] Selye, Hans, and Schenker, Victor, PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 518.

tions, the former in mg, the latter in g/100 ml of whole blood.

It appears that the high dose of our potent adrenal cortical extract failed to alter the blood sugar level in the intact animal. In the adrenalectomized but not traumatized rat, it merely restored the low initial blood sugar to the normal level. This confirms previous observations showing that corticoid hormones are unable to raise the blood sugar above normal under these experimental conditions. It shows, furthermore, that our extracts did not contain any significant amount of adrenalin. In the traumatized adrenalectomized animals, the blood sugar did not show the usual hyperglycemia which is caused by a similar degree of trauma in the presence of the adrenals. Indeed the average blood sugar declined slightly after the intervention. On the other hand, the corticoid hormone treated adrenalectomized rats developed a distinct hyperglycemia (more than 40 mg % on the average) during the development of traumatic shock. That the trauma was sufficiently severe to cause shock was evident both from the clinical condition of the animals and from the rise in blood hemoglobin shown in our table. Judged by these two criteria, the corticoid extract-treated traumatized animals were not nearly as severely damaged as the untreated traumatized controls.

It appears from these experiments that an adrenal cortical extract capable of raising the shock resistance of the adrenalectomized rat, causes a significant elevation of the blood sugar when it is given after trauma. Since neither trauma alone, nor the extract alone, were able to raise the blood sugar after adrenalectomy, it is evident that the action of corticoid hormone on the glucose concentration of the blood is in some manner modified by tissue damage. Thus traumatic shock so modifies the action of corticoid hormones that they induce hyperglycemia. This observation is equally compatible with the toxic and with the deficiency theory of shock, since the change in responsiveness may be due either to the presence of an abnormal metabolite or to deficiency in a normal body constituent.

Summary. Experiments in the rat indicate that trauma fails to elicit the usual hyperglycemia in the absence of the adrenals. Active corticoid hormone preparations restore the low blood sugar of adrenalectomized animals to normal, but do not raise it above this level, nor do they cause hyperglycemia in intact animals. If such extracts are administered to adrenalectomized rats during traumatic shock, they cause a significant rise in the blood sugar concentration and at the same time increase shock resistance. It appears that the metabolic changes elicited by extensive tissue damage alter the action of adrenal cortical hormones on the blood sugar.

Since lactic acid production is increased in traumatic shock[6] and corticoids raise the blood sugar only in the presence of the liver,[3] it is tempting to assume that in shock, the blood sugar decreases in the absence of the adrenals because the lactic acid produced fails to be resynthesized into glucose. If, on the other hand, carbohydrate-metabolism-active corticoids are administered, the blood sugar can be raised above normal by resynthesis of the excess lactic acid. In any case, these experiments give further support to the assumption that changes in carbohydrate metabolism play an important part in the pathogenesis of the shock syndrome.

13381

Gas Gangrene-Toxin Production in Gelatin-Thioglycollate Medium.*

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In a recent note, Reed, Orr and Baker[1] showed that good yields of *Cl. welchii* toxin were produced in a simple gelatin-peptone medium. Since that work was done a further simplification of the medium has been found to give larger and more consistent yields of both hemotoxin and lethal toxin by the 4 principal gas gangrene species: *Cl. welchii, Cl. septicum, Cl. novyi* and *Cl. sordellii.*

1. This simplified medium was based on the observation of Koser, Chinn and Saunders[2] that certain brands of gelatin support growth of many of the more exacting aerobic species.

The growth of several species of the genus *Clostridium* was tested in a medium in which gelatin provided the only source of nitrogen. Four brands were tried. Five percent concentration of the 4 gelatins were dissolved in the following salt-sugar solution:

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[6] Dosne, C., Proc. of the 7th Annual Meet., Canad. Physiol. Soc., Montebello, October, 1941.

* Part of an investigation aided financially by the Canadian National Research Council.

[1] Reed, G. B., Orr, J. H., and Baker, M. C., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 620.

[2] Koser, S. A., Chinn, B. D., and Saunders, F., *J. Bact.*, 1938, **36**, 57.

Na_2HPO_4	5	g
KH_2PO_4	1	"
NaCl	2	"
$MgSO_4$	0.1	"
Dextrose	2	"
Water	1000	ml

The reaction was adjusted to pH 7.6 and the media autoclaved in deep tubes.

Table I indicates that of the 4 gelatins tested, Difco and Grayslake (a domestic brand) support abundant growth of the 6 more important species of gas gangrene anaerobes. Eastman's "practical" permits a slight amount of growth in the first culture but none in subsequent transfers. Eastman's "purified gelatin (de-ashed)" fails to support growth of any of the 6 species. This corresponds with Koser, Chinn and Saunder's observations on aerobes, that highly purified photographic gelatin lacks some growth-promoting factor or factors present in certain laboratory and domestic gelatins.

TABLE I.
Comparative Growth Promoting Effect of 4 Brands of Gelatin in 5% Concentrations in a Salt-Sugar Solution.

	Difco		Grayslake		Eastman practical		Eastman purified	
	1st gen.	2nd gen.	1st gen.	2nd gen.	1st gen.	2nd gen.	1st gen.	2nd gen.
Cl. welchii	+	+	+	+	±	—	±	—
Cl. septicum	+	+	+	+	—	—	—	—
Cl. novyi	+	+	+	+	—	—	—	—
Cl. sordellii	+	+	+	+	—	—	—	—
Cl. histolyticum	+	+	+	+	—	—	—	—
Cl. sporogenes	+	+	+	+	±	—	±	—

2. The medium which has given most consistent yields of toxin is made as follows:

Gelatin (Bacto or Grayslake)	100	g
Na_2HPO_4	5	"
KH_2PO_4	1	"
NaCl	2	"
$MgSO_4$	0.1	"
Dextrose	2	"
Na thioglycollate	1	"
Water	1000	ml

The ingredients are dissolved together, adjusted to pH 7.6 before autoclaving, dispensed in tubes or flasks to a depth of not less than 6 cm and autoclaved at 15 lb for 25 minutes, in the case of tubes, or longer for larger containers.

Table II indicates the yields of hemotoxins and lethal toxins from the four principal toxigenic gas gangrene species. In this table the

TABLE II.
Hæmotoxin and Lethal Toxins Produced in Gelatin-Na-thioglycollate Medium.

	Hæmotoxin, active dilution	Lethal toxin, M.L.D. mouse
Cl. welchii	1-1020 to 1-480	0.5 to 1.0 ml
Cl. septicum	1-32 " 1-128	0.1 " 0.01 "
Cl. novyi	1-32 " 1-128	0.1 " 0.01 "
Cl. sordellii	1-4 " 1-16	0.1 " 0.01 "

figures for hemotoxin represent the highest dilution of the culture filtrate which will hemolyze 50% of a 2% suspension of washed rabbit red cells. The figures for lethal toxin represent the smallest volume of culture filtrate which kills a 20 g white mouse, after intraperitoneal injection in 5 to 7 hours. The range indicated in the table represents yields from several strains of each species mentioned. Any one strain maintained by day to day transfers in this gelatin medium shows very little variation in toxin yield on successive trials in the same batch or on different batches of the medium.

Several old laboratory strains of *Cl. welchii, Cl. septicum* and *Cl. novyi* which yielded negligible amounts of toxin gave good yields after 8 to 10 mouse passages.

The toxins produced in the gelatin medium are actively antigenic. Highly potent antitoxins have regularly been obtained in rabbits, after 8 to 10 doses of toxin. One ml of these antitoxins will neutralize 1000 or more lethal doses of the specific toxin, tested in white mice.

Ten days' treatment of the toxins with 0.3% formalin results in loss of toxicity. The resulting toxoids are as active or more active than fresh toxins in stimulating antitoxin formation in the rabbit.

Both the hemotoxin and the lethal toxin may be recovered by centrifuging at high speed, or by filtration through Seitz or sintered glass (Jena G5 on 3) filters. Filtration results in only a very slight decrease in toxin content, especially if the first 20 to 30 ml of toxin, in the case of small filters, are discarded.

3. In this medium the growth and toxin yield are closely related to the initial oxidation-reduction potential, which in turn is influenced by the concentration of gelatin and, of course, by the addition of reducing agents. Fig. 1 indicates the oxidation-reduction potential of media with 2 to 20% gelatin, with and without 0.1% sodium thioglycollate, before and after inoculation with *Cl. welchii.* It is apparent that the Eh of the medium, without thioglycollate, is progressively more negative with increasing concentration of gelatin. On the other hand, the addition of 0.1% sodium thioglycollate brings the several concentrations of gelatin to approximately the same Eh.

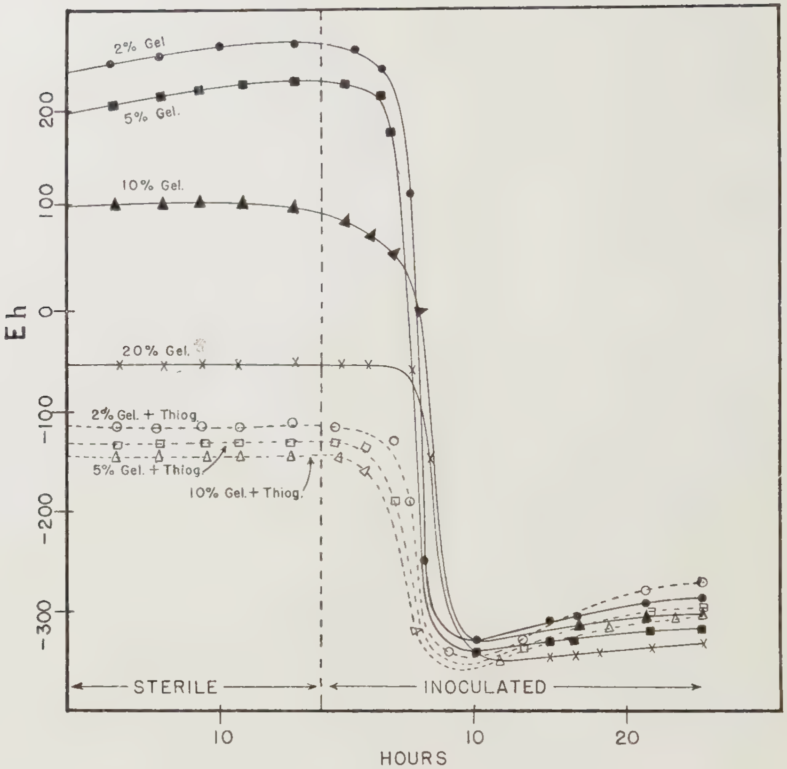

FIG. 1.

Graphs showing the Eh of gelatin medium, 2 to 20% gelatin, with and without sodium thioglycollate, before and after inoculation with *Cl. welchii*.

When heavily inoculated with *Cl. welchii* (0.01 ml of an 18-hour culture in 5% gelatin medium with thioglycollate) the fall in potential is similar in all the media, with and without thioglycollate. Ascorbic acid and cysteine in 0.1% concentrations affect the potential in essentially the same manner as sodium thioglycollate.

The influence on the medium of gelatin concentration and added reducing agents from the point of view of growth is indicated by the minimum effective inoculum of *Cl. welchii*, shown in Table III. It is apparent that in the absence of added reducing agent, growth is promoted by increasing the concentration of gelatin; this runs parallel with the more negative potential effected by the higher gelatin concentration. However, when the potential is rendered still more negative with sodium thioglycollate, the low gelatin concentrations still do not provide an efficient medium. It is, therefore, apparent that the higher gelatin concentrations provide some growth-promoting or nutritive factor in addition to shifting the Eh toward a more

TABLE III.

Minimum Inoculum of *Cl. welchii* to Initiate Growth in Gelatin Media with 2 to 20% Gelatin With and Without Sodium Thioglycollate.

Inoculum, ml. *Cl. welchii* culture	2% gelatin thioglycollate		5% gelatin thioglycollate		10% gelatin thioglycollate		20% gelatin thioglycollate	
	none	0.1%	none	0.1%	none	0.1%	none	0.1%
10^{-3}	+	+	+	+	+	+	+	+
10^{-4}	—	+	+	+	+	+	+	+
10^{-5}	—	—	—	+	+	+	+	+
10^{-6}	—	—	—	—	+	+	+	+
10^{-7}	—	—	—	—	—	+	—	+
10^{-8}	—	—	—	—	—	+	—	+
10^{-9}	—	—	—	—	—	—	—	—

+ indicates definite growth in 24 hours; — = no growth.

favorable level. It is further evident that the medium with 2 to 10% gelatin is more readily inoculated with *Cl. welchii* when the Eh is lowered to approximately 100 millivolts by the addition of sodium thioglycollate. Similar effects on growth are produced by the addition of cysteine or ascorbic acid.

The gelatin concentration in the medium also conspicuously influences toxin yield, as indicated in Table IV. Two and 5% concentrations give relatively low yields, 10 and 20% give much higher yields. This is probably proportional to growth. The addition of sodium thioglycollate increases toxin production in low concentrations of gelatin but has little influence in the higher concentrations provided the inoculum is large enough to initiate rapid growth.

TABLE IV.

Yield of Hæmotoxin in Medium with Increasing Concentrations of Gelatin, with and without Sodium Thioglycollate.

Medium	Hæmotoxin dilution					
	1-10	1-100	1-1000	1-2000	1-4000	1-8000
2% gelatin						
No thioglycollate	+	—	—	—	—	—
0.1% ”	+	+	—	—	—	—
5% gelatin						
No thioglycollate	+	+	—	—	—	—
0.1% ”	+	+	+	+	—	—
10% gelatin						
No thioglycollate	+	+	+	+	+	+
0.1% ”	+	+	+	+	+	+
20% gelatin						
No thioglycollate	+	+	+	+	+	+
0.1% ”	+	+	+	+	+	+

+ indicates haemolysis of 50% or more of a 2% suspension of red cells.
— indicates no haemolysis or less than 50% of the 2% red cell suspension.

Conclusions. The gas gangrene group of anaerobes grow luxuriantly in a medium consisting of a buffered salt-mixture with dextrose and gelatin. Highly purified gelatin is not suitable. In this medium all the toxigenic gas gangrene species produce a high and consistent yield of hemotoxin and lethal toxin. The toxins are actively antigenic and make effective toxoids.

13382

Effect of Quinine on Metabolism in Fasting Dogs and in Patients with Creatinuria.*

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The observation that quinine diminishes the output of certain urinary substances has been confirmed by several investigators; however, practically all of the experiments heretofore reported were made on animals or human subjects receiving an adequate diet. Prior[1] appears to have been the only investigator who studied these effects of quinine during fasting and in his studies, which were made on only one dog, urea was the only urinary substance that was determined. Aoki[2] found a diminution in the output of creatine and creatinine when quinine was given to patients with hyperthyroidism and animals which had received desiccated thyroid gland. Since quinine is now used in the management of certain muscular disorders, *e. g., myotonia congenita* and muscular rigidity of paralysis agitans, it seemed of interest to further investigate the metabolic effects in an attempt to elucidate the action of the drug in these muscular syndromes.

Methods. There were two series of experiments, one on fasting dogs, the other on patients with muscular wasting and creatinuria. Female dogs were fasted for a preliminary period of at least 10 days and throughout the experiments but were allowed free access to drinking water. The animals were kept in metabolism cages

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* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Prior, *Arch. f. d. ges. Physiol.*, 1884, **34**, 237.

[2] Aoki, K., *Folia endocrin. Jap.*, 1928, **3**, 1331 (abstr. *Jap. J. Med. Sci.*, 1931, **4**, 13).

which permitted quantitative collection of all voided urine specimens. At the end of each 24-hour period the animals were catheterized and the bladder rinsed with sterile distilled water.

After a control period of several days during which the urinary output of the various substances was constant, each animal was given quinine by mouth in doses of from 0.2 to 0.4 g. In some experiments a single dose was given; in others the drug was administered on successive days for periods up to 6 days. Twelve complete experiments on 4 dogs were made.

The patients were 2 boys, aged 10 and 13 years, with progressive muscular dystrophy of several years' duration. Both patients showed considerable wasting and weakness of most of the muscles of the trunk and extremities. The subjects were maintained in a special metabolism ward where the diet could be rigidly supervised, and where quantitative collections of urine and feces could be made.

For a preliminary period of a few weeks and during the periods of observation the diet was kept constant from day to day. Both patients were given amounts of quinine which appeared to be as much as they were able to tolerate without significant side-effects.

Observations and Discussion. In fasting dogs the administration of quinine produced significant decreases in the excretion of total nitrogen, urea nitrogen, phosphorus and inorganic sulfur, whereas the elimination of creatine, creatinine, vitamn C, ammonia, amino-acid nitrogen and organic sulfur were unaffected. The output of chlorides which was determined in only one experiment was definitely lowered after quinine administration. The effect of quinine was variable during the first day after the drug had been given. In the main, there was but little effect on the elimination of the urinary constituents during the first day in most experiments, but on the second and third day following the administration of quinine the decrease in nitrogen excretion amounted to as much as 30% in some experiments. The effect of quinine appeared to be more definite when a single dose of the drug was given than in the experiments in which quinine was given on several successive days. The greatest effect of quinine was observed when the urine no longer gave a positive test for the drug with Mayer's reagent. Since the urinary elimination of nitrogen and other substances often was increased on the first day after the drug had been given it would appear that quinine first increases the elimination of nitrogen, urea, and phosphorus and then later decreases the output of these substances. This would explain why the effect of the drug on nitrogen output is more apparent when a single dose of quinine is given than when the drug is given on successive days. In other words, the late effect of the drug in decreasing the urinary nitrogen often is partially offset by the subsequent doses, the early effects of which would be to increase the output. In experiments in which the drug was given for 6 consecutive days no increase in the effect was noted after the second day. When the administration of the drug was stopped no subsequent change in the nitrogen output was observed, suggesting that the level of nitrogen elimination on the sixth day of quinine administration was no lower than that induced by continuance of fasting alone.

The data on patients are given in Tables I and II and are in essential agreement with those on fasting dogs. Both patients had a slightly increased output of urinary nitrogen on the days when quinine was administered. In one subject (Case 2), in whom the excretion of fecal nitrogen was determined, the retention of nitrogen was reduced during the period when the drug was given, and increased in the subsequent period of observation. Since the elimination of nitrogen in the stool was essentially unchanged, the alterations in urinary output could not have been the result of changes in the intestinal absorption of the nitrogen.

TABLE I.
Effect of Quinine on Urinary Creatinine and Creatine.

Patient	Nitrogen intake, g	Daily urinary output Nitrogen, g	 Creatinine, g	 Creatine, g	Quinine sulfate *per os*, g
1	8.00	4.73	.200	.382	
	8.16	5.43	.200	.376	
	8.16	5.02	.200	.437	
	8.80	5.93	.198	.376	0.4
	8.16	6.02	.200	.437	0.4
	8.16	5.88	.195	.367	
	8.00	6.06	.193	.407	1.0
	8.00	5.55	.198	.440	
	8.16	6.08	.171	.404	1.4
	8.16	5.12	.184	.451	
	8.00	6.18	.184	.423	1.6
	8.00	4.58	.180	.378	
2	Data in Table II		.178	.497	
			.167	.565	
			.178	.490	
			.149	.469	0.6
			.162	.486	0.6
			.203	.474	0.6
			.180	.510	1.0
			.151	.462	1.0
			.178	.480	
			.180	.503	
			.186	.529	

TABLE II.
Effect of Quinine on Daily Retention of Nitrogen, Calcium, Phosphorus and Inorganic Sulfur in Case 2.

Daily output												
Nitrogen		Calcium		Phosphorus		Inorg. sulfur		Daily retention				Quinine sulfate, *per os*, g
Urine, g	Stool, g	Urine, g	Stool, g	Urine, g	Stool, g	Urine, g	Stool, g	N, g	Ca, g	P, g	S, g	
5.53	1.03	.043	.743	.557	.390	.295	.013	1.62	.343	.281	.238	
5.47	1.03	.046	.743	.568	.390	.282	.013	1.68	.340	.270	.251	
5.40	1.03	.051	.743	.545	.390	.264	.013	1.75	.333	.293	.269	
5.25	1.12	.042	.822	.520	.464	.292	.013	1.81	.265	.284	.287	.6
5.78	1.12	.031	.822	.593	.464	.340	.013	1.28	.275	.171	.239	.6
5.60	1.12	.032	.822	.483	.464	.329	.013	1.46	.275	.281	.250	.6
6.22	1.12	.032	.822	.502	.464	.395	.013	0.84	.275	.262	.234	1.0
5.78	1.12	.026	.822	.450	.464	.329	.013	1.28	.281	.314	.295	1.2
5.40	1.36	.025	.967	.440	.483	.258	.021	1.42	.137	.295	.267	
4.98	1.36	.034	.967	.500	.483	.214	.021	1.84	.128	.245	.311	
4.83	1.36	.042	.967	.566	.483	.197	.021	1.99	.120	.179	.328	

The daily intakes of nitrogen, calcium and phosphorus were constant and were 8.18 g, 1.129 g and 1.228 g, respectively. The intake of sulfur was 0.546 g daily except on days of quinine administration when the intake was 0.592 g for first three days, 0.622 g on fourth day and 0.637 g on fifth day.

The urinary elimination of calcium was unchanged, but the amounts in the feces were increased during both the period when quinine was given and in the subsequent period, so that the retention of calcium was reduced during both these periods. Likewise, the fecal elimination of phosphorus was increased during these two periods, with the result that the retention was slightly diminished during the period of drug administration and during the subsequent period, although the urinary output was decreased.

Of interest are the findings on the urinary outputs of preformed creatinine and creatine. In contrast with the observations of Aoki[2] no significant change in the output of these substances was observed in the present investigations. These findings offer support to the interpretation that the effect of quinine on muscular function in certain disorders is not due to a direct metabolic action on the muscles. In a previous report[3] it was postulated that the effect of quinine in muscular disorders is due to an antagonistic action on cholinergic nerve stimulation.

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[3] Milhorat, A. T., *Arch. Neurol. and Psychiat.*, 1941, **45**, 74.

13383

Effect of Propylene Glycol Aerosol on Air-Borne Virus of Influenza A.

WERNER HENLE AND JOSEPH ZELLAT. (Introduced by Stuart Mudd.)

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The recovery of the virus of influenza A from artificially infected air[1] showed that air-borne transmission of this agent is possible. Exposure of ferrets to infected air proved that the virus can be transmitted by this route.[2, 3] The development of a new technic for the study of experimental air-borne infection[4] permitted the exposure of ferrets and mice to the infected air on a quantitative basis and typical disease followed the inhalation of the air-borne influenza virus.[5] It was found that ultraviolet irradiation of the infected air destroyed the virus[1] and disease did not develop in animals exposed to air-borne virus thus treated.[5]

Sterilization of air by aerosols has been attempted, using various bacteria and fungi as test organisms[6–9] but only a preliminary statement regarding their effect on viruses has been published.[9] In a recent study Robertson and his coworkers[10] demonstrated the successful application of glycols as aerosols to the destruction of bacteria suspended in air as shown by plating methods. The present paper adds the virus of influenza A to the group of infectious agents attacked by propylene glycol aerosols. The direct animal test, *i. e.*, the exposure of mice to the infected air treated with this reagent was the experimental method used.

Briefly the technic employed was as follows: One atomizer flask[4] contained the virus suspension, another either propylene glycol or water as control. The air-streams from both of the atomizer flasks carrying the atomized materials were mixed in a 5 liter bottle and the mixture was introduced into the infection chamber.[4] The air was forced through the system instead of being aspirated by an incinerating chimney. With 0.2 to 0.3 cubic foot of air passing through the apparatus per minute a complete turnover of the air was accomplished in 1½ to 2 minutes.

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[1] Wells, W. F., and Brown, H. W., *Science*, 1936, **84**, 68; *Am. J. Hyg.*, 1936, **24**, 407.

[2] Trillat, A., and Beauvillain, E., *Compt. rend. Acad. d. Sci.*, 1937, **205**, 1186; *Rev. d'Hyg.*, 1938, **60**, 104.

[3] Andrewes, C. H., and Glover, R. E., *Brit. J. Exp. Path.*, 1941, **22**, 91.

[4] Wells, W. F., *Science*, 1940, **91**, 172.

[5] Wells, W. F., and Henle, W., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 298.

[6] Trillat, A., *Bull. de l'Acad. de Med.*, 3 Serie, 1938, **119**, 64.

[7] Pulvertaft, R. S. V., and Walker, J. W., *J. Hyg.*, 1939, **39**, 696.

[8] Twort, C. C., Baker, A. H., Finn, S. R., and Powell, E. O., *J. Hyg.*, 1940, **40**, 253.

[9] Andrewes, C. H., *Lancet*, 1940, **2**, 770.

[10] Robertson, O. H., Bigg, E., Miller, B. F., and Baker, Z., *Science*, 1941, **93**, 213.

The extra-embryonic fluids of the chick infected with influenza A[11] served as virus suspension. In some experiments fluids previously kept at –72°C were used and repetition produced closely similar results. Only the F-12 strain[12] was employed in this study. White mice were exposed for 45 minutes to the infected air and the lesions following the inhalation of the air-borne influenza virus in this set-up resembled those described elsewhere.[5]

The effect of an aerosol of propylene glycol on influenza virus suspended in air is demonstrated in Table I. Serial decimal dilutions of the virus were atomized, and the infected air was mixed either with the propylene glycol aerosol or with atomized water as control. The concentration of either agent in the air was approximately 1 part in 200,000, as determined by the loss of weight from the atomizer flasks during the experiment. The control group exposed to the atomized 10^{-3} dilution of the virus showed lesions in more than 50% of the mice, while no lesions were observed in the experimental animals when a 10^{-2} dilution of the virus was atomized in the presence of propylene glycol. A reduction in active virus of 90 to 99% was therefore indicated by these results. One reaches a similar conclusion by comparing the death rate in the 2 groups of mice. While in the control group the atomized 10^{-1} dilution of virus produced death in nearly 50% of the animals, only one mouse in the experimental group died following inhalation of the highest concentration of virus. In other experiments a concentration of propylene glycol in air of 1 part in 1 million still proved effective in reducing the air-borne virus. Using a very strong virus preparation the only effect of the propylene glycol aerosol noticeable was a prolongation of the time of survival. No attempt was made to reduce the turnover of air which was accomplished every 1½ to 2 minutes, to allow for a more extensive interaction between the air-borne virus and the aerosol.

Propylene glycol neutralized the virus when added to it in equal

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[11] Henle, W., and Chambers, L. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 713.

[12] Stokes, J., Jr., and Wolman, I. J., *New International Clinics*, 1940, **1**, 115.

TABLE I.

Effect of Propylene Glycol Aerosol upon Air-borne Influenza Virus.

Flask 1 Aerosol	Flask 2 Dilution of virus	Air-borne inoculation					Intranasal inoculation of wash fluid			
		Influenzal lesions in mice								
Propyl. glycol	undil.	D11	3	2	2	1	0	0	0	0
		1	1	0	0	0				
	10^{-1}	2	2	2	1	1	0	0	0	0
		1	1	0	0	0				
	10^{-2}	0	0	0	0	0	0	0	0	0
		0	0	0	0	0*				
Water	undil.	D6	D6	D6	D8	D8	D5	D8	D8	2
		D8	D9	D9	D9	D11				
	10^{-1}	D9	D9	D9	D11	4	D7	D9	D10	3
		3	3	3	3	2				
	10^{-2}	3	2	2	2	2	2	1	1	0
		2	1	1	1	1				
	10^{-3}	2	2	1	1	1	0	0	0	0
		1	0	0	0	0				
	10^{-4}	0	0	0	0	0	0	0	0	0
		0	0	0	0	0				

D5 = died on the fifth day.
4 = lung totally consolidated when autopsied on 11th day.
3 = ¾, 2 = ½, 1 = ¼ of the lung consolidated.
* = atypical death with no lung lesion.

proportions. This was shown by intranasal instillation of the mixture into slightly anesthetized mice. While undiluted propylene glycol neutralized one million MLD of virus or possibly more, a 10% solution was without effect. Under the conditions of the experiment, however, the virus came into contact with the undiluted propylene glycol as an aerosol[10] or vapor, which therefore proved effective.* When the mixing bottle was washed with 2 or 3 ml of broth to collect traces of fluid precipitated during the experiment, this wash fluid was not infective when tested in mice by the intranasal route, unless the dosage of virus used in the experiment was overwhelmingly large. The wash fluid from the control experiments produced typical lesions in the 3 most concentrated dilutions atomized (Table I). Inhalation of a propylene glycol aerosol prior to an intranasal instillation of 10 MLD of virus did not protect the experimental animals.

From these experiments it can be concluded that propylene glycol aerosol reduces the chance of air-borne infection with the virus of influenza A and may be effective in preventing air-borne spread of the disease. The practicability of its use for this purpose has to be investigated.

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* Dr. Robertson (personal communication) suggested that the propylene glycol aerosol acts by means of liberation of vapor from the droplets.

We are indebted to Dr. O. H. Robertson for reading and criticising this manuscript. Experiments conducted in Dr. Robertson's laboratory also led to the conclusion that propylene glycol is effective in combatting the air-borne influenza virus.[13]

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[13] Robertson, O. H., Read before Section N, A. A. A. S., Chicago, September 23, 1941, to be published under the auspices of the A. A. A. S. as a symposium-monograph.